NUCLEAR FUNCTIONS OF ADENOMATOUS POLYPOSIS COLI: REGULATION OF THE G2-M CELL CYCLE TRANSITION & INTERMEDIATE FILAMENT INTERACTION

BY

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Abstract

Mutation of tumor suppressor *Adenomatous Polyposis Coli* (*APC*) is an early, if not the first step in the majority of colorectal cancers. As a multi-functional protein, APC has been implicated in regulating cell cycle and cytoskeletal integrity. To further understand APC function in cell cycle regulation, I immunoprecipitated APC and identified novel associated proteins. I identified topoisomerase IIα (topo IIα), a critical regulator of the G2 decatenation checkpoint, as a potential binding partner of APC. The interaction of endogenous APC and topo IIα was verified by co-immunoprecipitation, co-localization, and Förster resonance energy transfer (FRET). Both the fifteen (M2-APC) and twenty (M3-APC) amino acid repeat regions of APC interacted with topo IIα when expressed in cultured cells. Cells expressing M2- or M3-APC arrested in G2, but only if the cells contain normal levels of topo IIα. This G2 cell cycle arrest likely resulted from reduction of endogenous topo IIα activity, consistent with the established model whereby inhibition of topo IIα activates the G2 decatenation checkpoint.

APC has been shown to interact with microtubules and the actin cytoskeleton, inplicating APC in cell polarity and migration. Using a novel APC antibody raised against M2-APC, I co-immunoprecipitated potential APC binding proteins from HCT116βw cells. I identified 42 proteins in complex with APC by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). Among these were intermediate filament (IF) proteins lamin B1 and keratin 81. Lamin B1 interacts with APC in both cultured cells and human colonic tissue. APC also associated with IF

proteins throughout a sequential extraction procedure, which removed actin microfilaments and microtubules.

Based on evidence described in this dissertation, I hypothesize that two central APC domains interact with topo IIα to regulate the G2 decatenation checkpoint. In addition, I also provide evidence supporting a role for APC in the regulation of cytoskeletal integrity.

Dedicated to my parents and my husband

给我亲爱的爸爸妈妈

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

AA	Amino acid
ACF	Aberrant crypt foci
APC	Adenomatous Polyposis Coli
ASEF	APC-stimulated guanine nucleotide exchange factor
ATR	ATM- and Rad3-related kinase
BMPR1A	Bone morphogenetic protein receptor, type IA
BRCA1	Breast cancer 1
BSA	Bovine serum albumin
β-TrCP	β-transducing repeat-containing protein
Cdc42	Cell division cycle 42
CHIP	Chromatin immunoprecipitation
CIN	Chromosomal instability
CKI	Casein Kinase I
CRCs	Colorectal Cancers
CtBP	C-terminal binding protein
DLG	Discs large
ES cells	Embryonic stem cells
FAP	Familial Adenomatous Polyposis
Fen-1	Flap endonuclease 1
FRET	Förster resonance energy transfer
GEF	Guanine nucleotide exchange factor

GFP	Green fluorescent protein
HDAC1	Histone deacetylase 1
HNPCC	Hereditary Nonpolyposis Colorectal Cancer
IB	Immuno blots
IC	Immunocytochemistry
IF	Intermediate filament
IHC	Immunohistochemistry
IP	Immunoprecipitation
JPS	Juvenile Polyposis Syndrome
KAP3	Kinesin superfamily-associated protein 3
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LP-BER	Long patch base excision repair
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MCR	Mutation cluster region
MIN	Microsatellite instability
Min	Multiple intestinal neoplasia
MMR	Mismatch repair
NES	Nuclear export signal
NGS	Normal goat serum
NLS	Nuclear localization signal
pAb	polyclonal antibody
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen

PCP	Planar cell polarity
PDZ	Post synaptic density discs large zonula occludens
PFA	Paraformaldehyde
Plk1	Polo-like kinase 1
PTP-BL	Protein tyrosine phosphatase –BL
Rb	Retinoblastoma
ROI	Regions of interest
RT	Room Temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard Error of the Mean
TA cell	transient amplifying cell
TBS	Tris-Buffered Saline
TCF/LEF	T-cell factor/lymphoid enhancing factor
TGFβRII	Transforming Growth Factor beta Receptor II
topo IIα	topoisomerase IIα
WRN	Werner helicase
x-corr	cross-correlation scores

CHAPTER 1

INTRODUCTION: COLORECTAL CANCER, ADENOMATOUS POLYPOSIS COLI & CELL CYCLE REGULATION

Part I. Pathogenesis of Colorectal Cancer

Colorectal cancer (CRC) is the third most common type of cancer and the second leading cause of cancer-related deaths in the United States. It is estimated that in 2008 there were 108,070 CRCs diagnosed and 49,960 deaths (1). CRC develops slowly in the terminal six to seven feet of the digestive tract, called the large intestine, which includes colon and rectum. The large intestine is responsible for absorption of water and mineral nutrients (1). The colon can be divided into two sections: the proximal colon, which connects to the small intestine; and the distal colon, which joins with the rectum. Benign lesions called adenomatous polyps can form from the intestinal epithelium. A small percentage of a these polyps eventually become cancers, called adenocarcinoma. Over 95% of all CRCs are thought to arise from adenomatous polyps (1).

The colonic epithelium

The intestinal lining contains three tissue layers: the innervated smooth muscle that executes peristalsis; the submucosa that contains connective tissue and mesenchyme; and

the mucosa, which is a sheet of epithelial cells (Figure 1.1). The epithelial layer is composed of three major cell types: absorptive enterocytes; enteroendocrine cells that secrete hormones; and goblet cells that secrete a protective mucus barrier. The epithelial layer invaginates into the submucosa, forming crypts. It is estimated that 4-6 stem cells reside at the bottom of each crypt (2, 3). Although the stem cells are predominantly quiescent, occasionally they divide and give rise to progenitor transient amplifying (TA) cells (Figure 1.1). These progenitor cells undergo continuous divisions. Each progeny from these progenitor cells is committed to a specific cell lineage. As progeny cells differentiate, they migrate toward the luminal surface of the gut. Whether these terminal differentiated enterocytes undergo apoptosis before they shed into the lumen is still controversial, although apoptotic cells have been found in the colonic crypts (4).

The intestinal epithelium continuously proliferates. The entire colonic crypt can repopulate in about 5 days (5). Therefore, a tightly regulated program that balances cell proliferation, differentiation, and cell death is required to ensure the integrity of the intestinal epithelium. Changes that cause imbalances in the system can contribute to tumor initiation. This exquisite control is not only programmed into the epithelium, but also in the adjacent mesenchyme, which send signals to epithelial cells that control proliferation, cell fate choices, terminally differentiation, and death (6, 7).

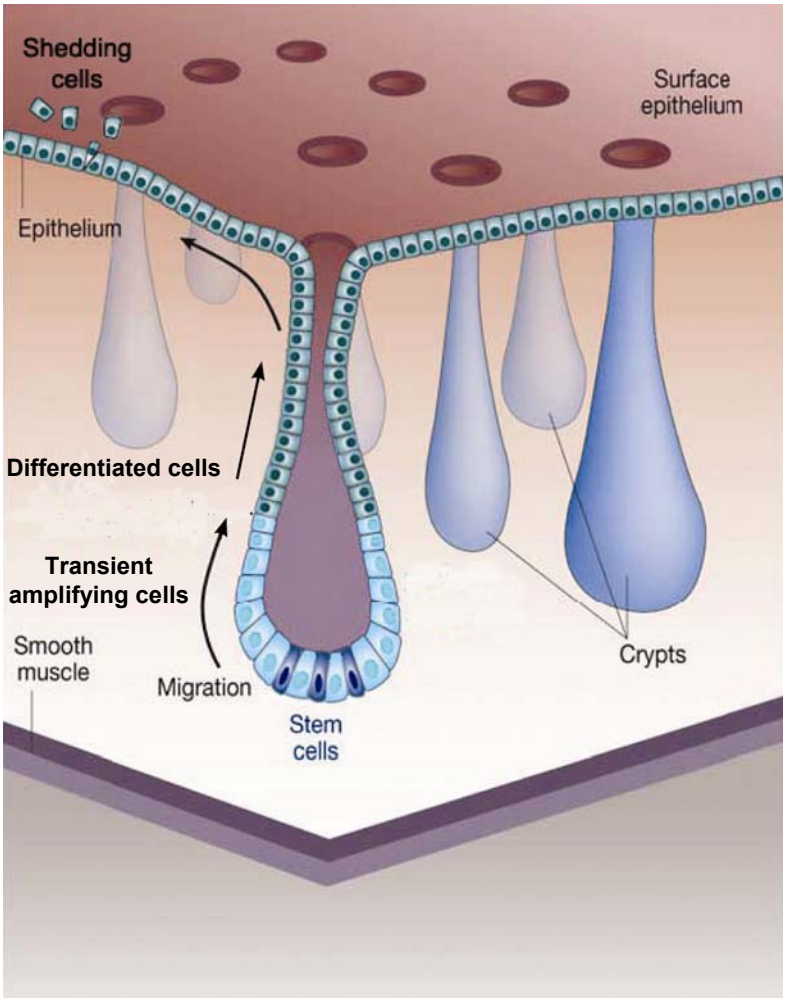

Figure 1.1

Structure of the colonic epithelia. Normal colon epithelia invaginates to form "crypt" structures. Each crypt comprises stem cells and transient amplifying (TA) cells in the progenitor compartment. As TA cells migrate up, they cease proliferation and begin to differentiate. Once TA cells reach the luminal surface, they are sloughed off into the lumen. (Adapted from Sancho and Clevers, 2004)

Molecular Pathogenesis of CRC

Although CRC was first described in the 18th century, the associated genetic defects were not revealed until the early 1990s (8). Inherited syndromes associated with CRC include Familial Adenomatous Polyposis (FAP), Hereditary Nonpolyposis Colorectal Cancer (HNPCC) and Hamartomatous Polyposis syndromes (9). FAP patients develop numerous adenomatous polyps and carry germline mutations in the *Adenomatous Polyposis Coli* (*APC*) gene (10-12). HNPCC patients develop fewer polyps and carry germline mutations in genes of the DNA mismatch repair (MMR) pathway (13, 14). Hamartomatous Polyposis syndrome is characterized by lesions involving mesenchymal tissues. One such syndrome, Juvenile Polyposis Syndrome (JPS), is associated with mutations in *SMAD-4 and* BMPR1A genes (15).

Half of the western population will develop at least one adenomatous polyp by the age of 70 (16). Mutation in *APC* is considered the first step in polyp formation and *APC* mutations are found in nearly 85% of all CRCs examined (17). CRCs with mutant APC are aneuploid and display chromosomal instability (CIN). In contrast, CRCs initiated by mutations in any of the five human MMR genes, *hMSH2, hMLH1, hMSH6, hPMS1* and *hPMS2* (13, 18-20) display microsatellite instability (MIN) (21). Microsatellites are tandem repeats of DNA sequences that are variable in length and found throughout the genome. In cells with mutations in DNA repair genes, microsatellites are abnormally

longer or shorter, referred to as microsatellite instability. HNPCC accounts for less than 2-4% of all CRCs (22). There are other CRCs associated with MIN. Some cancers with MIN posses mutations in other genes related to DNA repair such as *GTBP, MSH3* or *polymerase* δ (23-25). In addition to the CRCs with CIN and MIN, another ~5% of sporadic CRCs are thought to originate from sessile serrated adenomas with active mutation of the *BRAF* gene (26, 27).

Knudson established a "two-hit" hypothesis of tumor suppressor inactivation based on his statistical analysis of a type of childhood tumor that occurs in the retina, called retinoblastoma. Children with hereditary retinoblastoma inherit one mutant allele of the *Retinoblastoma* (*Rb*) gene (28). Mutation of the second *Rb* gene rapidly leads to cancer. Therefore, children with sporadic retinoblastoma develop cancer at an older age, because it takes longer to acquire mutations in both *Rb* genes than it does to develop a mutation in only the second *Rb* allele as required in the hereditary form. According to this "two-hit" tumor suppressor hypothesis, after the first *APC* gene is inactivated either in germline or in somatic cells, the second wild-type *APC* allele must also gain somatic mutation for APC to completely lose its function as a tumor suppressor (29, 30). Complete inactivation of APC has been observed in the majority of early human neoplastic lesions (30, 31). In mice with germline *Apc* mutations similar to those found in FAP patients, the wild-type *Apc* allele is always inactivated or lost in polyps (30). Whereas *APC* mutations are thought to initiate polyp formation; and mutations in genes other than *APC* are required

for tumorigenesis. It is believed that at least seven genetic mutations are required for tumorigenesis in human (32). Colorectal tumor starts from lesions called aberrant crypt foci (ACF), which are precursors of adenomatous polyps (32). Only a subset of ACF bearing *APC* and *K-RAS* mutations gives rise to adenomatous polyps that eventually progress to malignancy (33). In recent years, a "multiple hit hypothesis" has been proposed which suggests that multiple genetic mutations are required after an initial selective advantage to acquire malignant transformation.

Vogelstein proposed a classical model for colorectal tumorigenesis, in which at least three steps are required: initiation, promotion and progression (8). CRC results from inactivation of tumor suppressors in combination with activation of oncogenes (8). In FAP patients, germline mutations in APC are thought to be responsible for hyperproliferation, which allows additional mutations to accumulate (Figure 1.2A). Activating mutation of oncogene *K-RAS* in a preexisting adenoma can lead to a larger and more dysplastic tumor. Inactivating mutations in tumor suppressor *SMAD-4* and *Transforming Growth Factor Receptor II* (*TGFβRII*) may occur in some cells and thus provide a selective growth advantage to those cells. Loss of tumor suppressor gene *P53* often happens during the progression from adenoma to cancer. Although almost 85% of all CRCs are thought to initiate with mutations in *APC*, they do not necessarily share the classical order of these genetic events (mutations in *APC*, then *KRAS*, then *p53*) described in the "Vogelgram" (Figure 1.2A). It is thought that the accumulation of mutations in

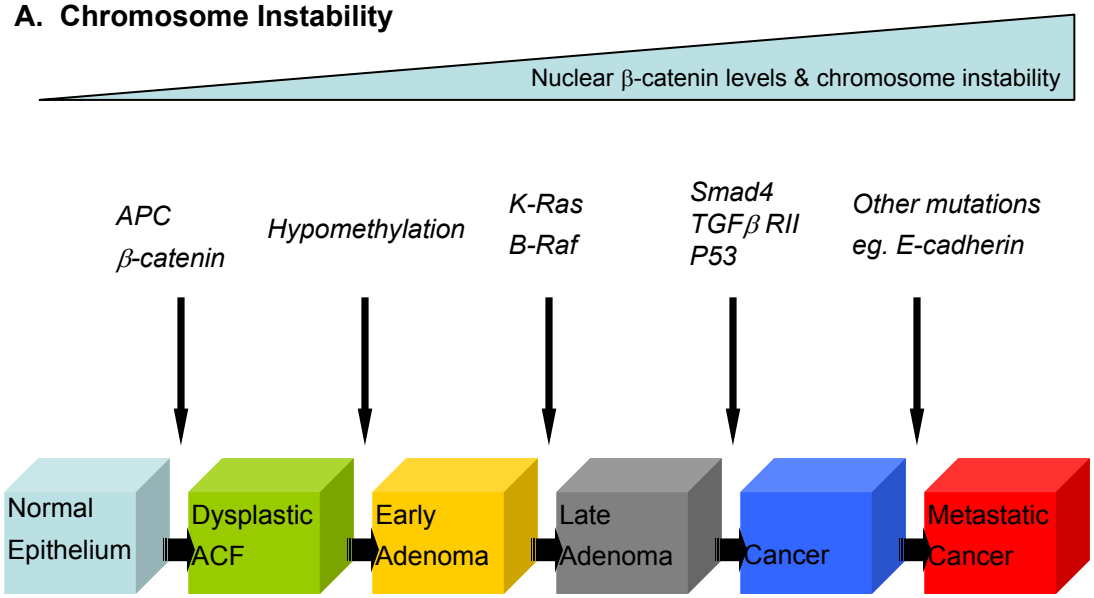

Figure 1.2

Sequential genetic changes in the pathogenesis of CRC. (A) Vogelgram of model for most FAP and some sporadic CRCs initiated from APC mutations (Adapted from Kinzler KW and Vogelstein B, 1996). A tumor evolves through a series of mutations of oncogenes and tumor suppressor genes. Only cells acquiring these mutations progress toward advanced lesions. (B) Events happening in HNPCC and many of the remaining sporadic CRCs. (Adapted from Voutsadakis, 2007) Epigenetic events such as CpG islands methylation happen in both models but are more frequent in (B).

key regulatory genes is more important for tumorigenesis than the exact order of these genetic events (8).

In another pathway that leads to HNPCC, mutations in DNA mismatch repair genes result in microsatellite instability (Figure 1.2B) (34). Defects in DNA repair cause alterations in the microsatellite DNA repeats resulting in variable repeat lengths, and frequently in codon frame shifts (35, 36). In addition, epigenetic changes, such as aberrant DNA methylation, can occur in early stages of CRC (37). Specific genes such as *hMLH1* and *BRAF* are often found to be inactivated by hypermethylation [reviewed in (38)]. These epigenetic events destabilize chromosomes and thus, potentially promote additional mutations.

Part II. Adenomatous Polyposis Coli

APC gene and protein

The human *APC* gene is located on chromosome 5q21-q22 and consists of 15 exons (10, 11). *APC* encodes a protein of 2843 amino acids (~312kDa) with multiple domains to which numerous proteins bind (Figure 1.3) (10, 11, 17). APC contains an oligomerization domain, an armadillo repeat-domain, a number of 15- or 20- amino acid (aa) repeats, a basic domain, and binding sites for end-binding protein 1 (EB1) and discs large (DLG). Most mutations of APC occur in a region called the mutation cluster region

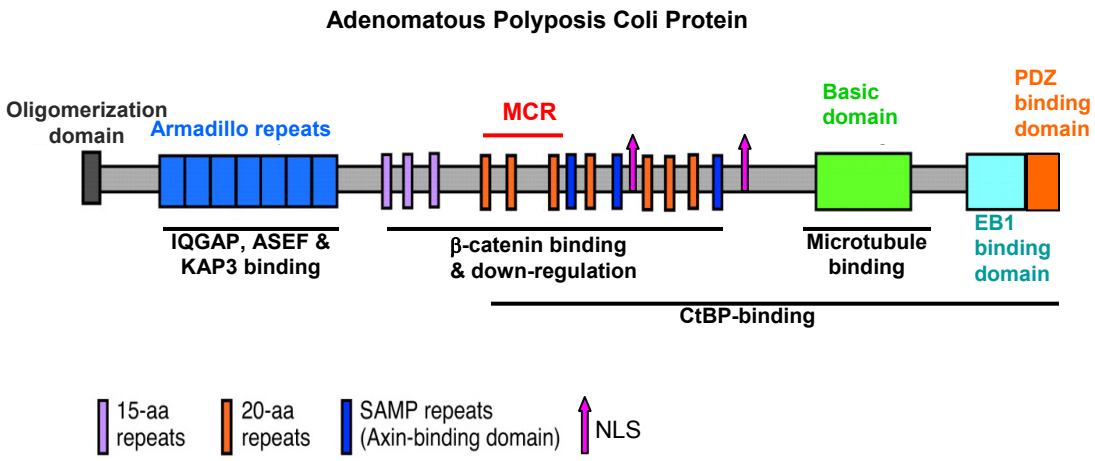

Figure 1.3

Structure of human APC protein. Multiple domains of APC are indicated in colors. Binding sites for APC-interacting proteins are indicated as well. N-terminal coil-coiled domain is responsible for APC oligomerization. Armadillo repeats mediate the interaction with IQGAP and ASEF. The 15-aa and 20-aa repeats are involved in β-catenin binding and down-regulation. SAMP repeats bind Axin. The basic domain binds to microtubules. The C-terminal domain binds to EB1 and DLG. (Modified from Aoki and Taketo 2007.)

(MCR) (39, 40). These mutations typically result in a premature translational stop between codons 1250 and 1450 and expression of just the N-terminal portion of the APC protein. The N-terminal coiled-coil domain of APC enables APC to form dimers (41). Both wild-type and truncated mutant APC are thought to form homo- or hetero-dimers (41, 42). The armadillo repeats of APC are nearly identical to regions of armadillo protein, the *Drosophila* homologue of mammalian β-catenin (11). There are three 15-aa repeats and seven 20-aa repeats in APC, which provide docking sites for β-catenin (43, 44). Although the 15-aa repeats are sufficient for β-catenin binding (43), the 20-aa repeats are required for β-catenin down-regulation (45). Contained within the 20-aa repeats are three SAMP repeat sequences, which mediate Axin-binding (46).

Within the C-terminus of APC there is a region comprising of predominantly basic amino acids (aa 2200-2400) (10). This basic domain and EB1 binding domain both bind to microtubules and can simulate tubulin polymerization *in vitro* (47, 48). The extreme C-terminal portion of APC contains a Post Synaptic Density Discs Large Zonula Occludens (PDZ) binding domain, including the STXV amino acid sequence. PDZ proteins DLG (49), protein tyrosine phosphatase–BL (PTP-BL) (50) and hScrib (51) have been reported to bind to the PDZ-binding domain of APC.

APC mRNA is highly expressed during embryogenesis (52). In adult animals, it is expressed in all tissues (53), with its highest expression in brain and colon (52). In normal human colonic tissue, APC protein predominantly localizes to the basolateral membrane

and cytoplasm of enterocytes, with some nuclear localization as well (17, 54, 55). Interestingly, the basolateral staining in colonic epithelial cells is increasingly pronounced as cells migrate from the bottom of the crypt toward the luminal surface (17, 53, 54, 56). Although some studies have reported apical plasma membrane localization of APC in mouse colonic enterocytes (57), it is now generally accepted that this staining represents protein(s) other than APC that are recognized by some commercial APC antibodies (58-60). In cultured colonic cells, APC has been detected in the cytoplasm and nucleus, at the plasma membrane, and at the tips of microtubules (61-64). This complex subcellular localization pattern of APC suggests that different pools of APC protein are involved in different cellular functions, such as cell adhesion and microtubule stabilization. Given the multiple functional domains of APC, its numerous binding partners, and the biological consequence of *APC* inactivation, it is likely that APC functions to integrate different signaling pathways to maintain cellular homeostasis.

APC in Wnt signaling

A major advance in our understanding of the role of APC in suppression of CRC came from the discovery of an association between APC and β-catenin (43, 44). APC forms a complex in combination with Axin and Glycogen synthase kinase 3β (GSK3β) to inhibit β-catenin activity to suppress the canonical Wnt signaling pathway (46). Canonical Wnt signaling plays vital roles throughout development (65). The central

component of Wnt signaling is β-catenin (gene name CTNNB1). In the presence of a soluble Wnt ligand, Wnt receptor Frizzled, together with co-receptor LRP5/6, blocks the binding and phosphorylation of β-catenin by the APC complex, leading to accumulation of β-catenin in the cytoplasm [reviewed in (65)] (Figure 1.4). Cytoplasmic β-catenin translocates into the nucleus, complexes with members of the T-cell factor/lymphoid enhancing factor (TCF/LEF) family of transcription factors to initiate transcriptions of Wnt target genes (66, 67), such as *Cyclin D1* and *MYC* (68). To suppress Wnt signaling, APC complex interacts with β-catenin (69, 70), phosphorylates β-catenin by GSK3β and Casein Kinase I (CKI) at the N-terminal Ser/Thr sites to promote its ubiquitination by E3 ubiquitin ligase β-transducing repeat-containing protein (β-TrCP) that targets it for proteosomal degradation (71). Wnt ligand also activates non-canonical Wnt signaling pathways, such as the planar cell polarity (PCP) pathway and a pathway involving Ca^{2+} signaling, for which we have limited mechanistic and functional information (72).

Although most truncated forms of APC retain part of the β-catenin binding domain, they lose regions critical for β-catenin destruction. As a result, human adenomas with *APC* mutations often display increased levels of nuclear β-catenin (73, 74). Analysis of several mouse models has confirmed that mutation of *Apc* leads to intestinal tumorigenesis (75-78). APC^{Min} (Multiple intestinal neoplasia) mice possess a wild-type and a mutant *Apc* allele, the later encoding a nonsense mutation at codon 850 (77, 78). $APC^{\Delta 716}$ mice produce a truncated APC protein containing the first 716 amino acids (75).

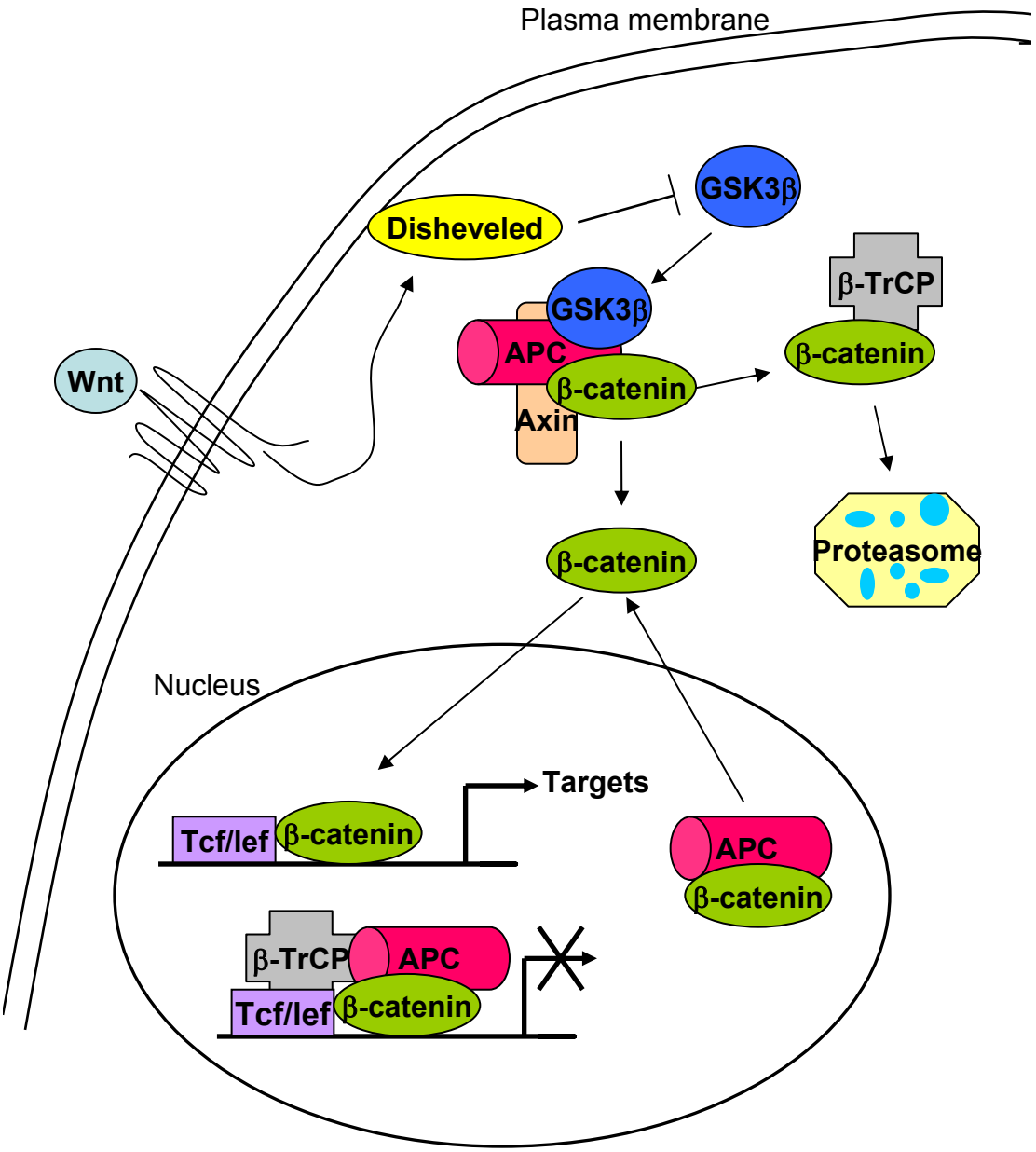

Figure 1.4

Model of APC regulation of β-catenin-mediated Wnt singling. Wnt ligands bind to frizzled receptor to activate disheveled, which inhibits phosphorylation of β-catenin and prevents its proteosomal degradation. Accumulated β-catenin translocates into the nucleus to activate downstream target genes. APC acts as a scaffold together with Axin and GSK3β to recruit β-catenin, and, target it for ubiquitination by β-TrCP and proteosomal degradation. In the nucleus, APC also inhibits β-catenin by sequestering it or by direct interacting with a repressor complex containing β-TrCP.

Mice from both models develop numerous polyps in their small intestines and display nuclear β-catenin as an indication of active Wnt signaling (76, 78). APC^{1638} mice, which express a less truncated APC protein, develop fewer tumors in the small intestine, thus are widely used for long term study of tumor progression (79). A more recently generated transgenic mouse model expressing a dominant stable β-catenin also develops small intestinal tumors, thus providing direct evidence that Wnt signaling is involved in intestinal tumorigenesis (80). Although less frequent than adenomas with mutant *APC*, human adenomas with mutant stabilized β-catenin but wild-type APC also exist (81). Nevertheless, tumors in mouse models with loss of APC function are found in multiple tissues, beyond those found in mice with activated β-catenin (80, 82). Although evidence provided from analysis of human adenoma and mouse models suggests the importance of Wnt signaling in colorectal tumorigenesis, APC appears to have functions beyond Wnt signaling.

Nuclear APC

APC has been observed to localize to the nuclei of both cultured cells (83) and mouse and human intestinal tissues (56, 84). In addition, both endogenous and epitope-tagged APC have been observed to localize to the nucleoli, although the APC function at this location is unclear (55, 83). Two nuclear localization signals (NLSs) have been identified in APC at aa 1767-1772 and aa 2048-2053 (85) (Table 1.1). APC also

possess five nuclear export signals (NESs) (Table 1.1) (55, 86, 87). These NESs and NLSs enable APC to shuttle between the nucleus and cytoplasm.

The nucleo-cytoplasmic shuttling of APC appears to facilitate APC inhibition of nuclear β-catenin activity by promoting its nuclear export (87-89) (Figure 1.4B). APC with mutated NESs is not able to efficiently target β-catenin for destruction, implicating APC in the nuclear export of β-catenin (87). However, both full-length APC and a central fragment of APC (aa 1379-2080) each with mutated NESs was able to down-regulate nuclear β-catenin activity, implicating nuclear APC in the sequestration of nuclear β-catenin (87, 88). A more recent study using chromatin immunoprecipitation (CHIP) demonstrated that APC binds to the *MYC* enhancer region in a repressor complex containing β-TrCP, C-terminal binding protein (CtBP) and histone deacetylase 1 (HDAC1) (90, 91). These studies provide evidence that APC inhibits Wnt signaling in ways other than by forming a cytoplasmic β-catenin destruction complex.

APC may directly bind to A/T-rich regions of DNA through clusters of S/TPXX located in the basic domain (92). In addition, APC also interacts with nuclear proteins involved in DNA repair. APC has been proposed to participate in long patch base excision repair (LP-BER) through interaction with DNA polymerase β and its cofactor proliferating cell nuclear antigen (PCNA) (93). APC blocks LP-BER by interacting with flap endonuclease 1 (Fen-1). Thus, APC is implicated in DNA damage-induced carcinogenesis (94).

Table 1.1 NLSs and NESs amino acid (aa) sequence and position information

NLS/NES	aa position	aa sequence
NLS1	1767-1772	GKKKKP
NLS2	2048-2053	PKKKKP
NES1	68-76	LLERLKELNL
NES2	165-173	LTKRIDSLPL
NES-R3	1506-1511	LSALSL
NES-R4	1657-1662	LSDLTI
NES-R6	2027-2032	LSSLSI

APC and cytoskeletal integrity

Cell proliferation, differentiation and migration synergize to maintain intestinal epithelial integrity [reviewed in section I]. The observation that APC can interact with the cytoskeleton suggests a role for APC in colonic cell architecture (95-97). APC has been reported to interact with microtubules and stabilize them by inhibiting depolymerization (47, 48, 98). APC has also been found to associate with EB1 at the basal cortex of polarized epithelial cells (99). Interaction of APC with actin filaments was inhibited by introducing purified recombinant EB1 *in vitro* (100). Disruption of the APC/EB1 interaction restored APC localization to actin (100). Colocalization of APC with EB1 on centrosomes also indicates a potential role for APC in the dynamics of microtubules and mitotic spindles (101, 102). In migrating cells, APC binds to EB1 at the microtubule plus end and thus potentially regulates microtubule integrity (97). APC lacking the EB1-binding domain binds microtubules throughout their length with no preference for microtubule plus end binding, suggesting the interaction of EB1 with APC is important for targeting APC to microtubule plus ends (103).

APC participates in the actin network by interacting with IQGAP, an activator for cell division cycle 42 (Cdc42) at the leading edge of migrating cells (95, 104). Depletion of either APC or IQGAP disrupts actin filament formation and polarized cell migration (95). APC also binds to a Rac specific GEF, APC-stimulated guanine nucleotide

exchange factor (ASEF), to stimulate actin polymerization (96). Interaction of APC with ASEF results in activation of Cdc42, thus suppressing anchorage-independent growth (105). Therefore, APC appears to stimulate cell polarization and suppress tumor formation through binding to IQGAP and ASEF and ultimately stimulating Cdc42.

APC is a junctional protein, colocalizing with β-catenin at cell adhesions (44). However, β-catenin, an adherense junction protein that interacts with E-cadherin, does not appear able to bind to both APC and E-cadherin at the same time (106). This exclusive binding suggests that the junctional APC/β-catenin interaction also participates in the suppression of tumorigenesis. Furthermore, forced expression of APC in a mouse model results in defects in cell migration, indicating the APC-β-catenin and E-cadherin-β-catenin complexes have opposing roles in epithelial movement (56). Besides β-catenin, APC was also reported to interact with DLG, a scaffolding protein associated with cell junctions (49). DLG contains three PDZ domains, which APC binds. APC/DLG interaction at the leading edge of epithelial cells is required for microtubule and basal membrane association (107).

Most mutations in *APC* result in expression of a truncated protein. This truncated APC loses direct binding sites for many protein partners such as EB1. Deletion of the PDZ-binding domain of APC abolishes APC plasma membrane localization and cell substrate attachment, therefore disturbing cell spreading and migration (108). In contrast, restoring full-length APC to colon cancer cells enhances cell adhesion (109). Truncated

APC lacks the PDZ binding domain, which provides a link to the actin cytoskeleton through DLG and other PDZ domain-containing proteins. Therefore, it has been hypothesized that *APC* mutations affect the cytoskeleton and thus, contribute to tumor initiation or progression.

So far, APC has been found to directly associate with two of the three major components of the cytoskeleton: actin filaments and microtubules. It will be of interest to study if APC also associates with the third component: intermediate filaments. My finding of APC interaction with IF proteins Lamin B1 and Keratin 81 linked APC to all three components of the cytoskeleton [see Chapter 4 for detail]. This study further supports functions of APC in regulating the cytoskeleton integrity thus preventing tumorigenesis.

APC and cell cycle regulation

Various studies support a role for APC in cell cycle regulation, especially in mitosis. In mitotic cells, APC localizes to the kinetochore, mitotic spindle and centrosomes (101, 110-112). However, it is still debated whether APC is required in the processes of spindle formation and chromosome segregation. Using *Xenopus* extracts, APC was shown to be required for spindle formation (110). Loss of APC resulted in kinetochore dysfunction and inhibition of apoptosis, leading to aneuploidy (113). Truncated APC fragments were shown to have dominant-negative effects on spindle formation, compromising

kinetochore-microtubule interactions and thus inducing CIN (114, 115). The observation that mutations in *APC* induce CIN supports the idea that APC functions in chromosome segregation (112, 115-117). On the other hand, others have reported that reduction of APC did not interfere with the spindle checkpoint or the stability of the kinetochore-microtubule interaction. However, APC reduction did cause chromosome mis-segregation (118). These differences may stem from distinctions between full-length APC and overexpressed truncated APC protein. It is also possible that APC functions in different pools. Only a small portion of full-length APC is required to maintain its function at kinetochore-microtubules. In contrast, ectopic expression of truncated APC protein might exhibit a dominant-negative effect. Nevertheless, germline *Apc* mutations in mice result in CIN, as evidenced by aneuploidy and chromosomal defects in embryonic stem (ES) cells and cells of polyps (112, 113, 117, 119). However, human adenoma studies failed to provide definitive evidence supporting the link between loss of APC and CIN (120). In addition, localization of APC at the midbody during cytokenesis has also been reported, although the APC function at this location is unclear (121).

Evidence suggests that APC functions throughout the cell cycle. Expression of APC in NIH3T3 fibroblast cells blocked the cell cycle in G1, by inhibiting transcription of Cyclin D1 through the Wnt signaling pathway (122). Expression of full-length APC in a colon cancer cell line with only endogenous truncated APC also resulted in G1 arrest (123). The APC-DLG complex has been implicated in this G1-S cell cycle arrest (124).

Mutant APC lacking the DLG binding site failed to block cell cycle in G1; however, the mechanism is unknown. A recent study showed that full-length APC negatively regulated cell cycle progression into or through S phase by inhibiting DNA replication (125). On the other hand, reduced expression of APC mRNA has been reported in cells undergoing G1 arrest induced by DNA-damaging agent MNNG (126). Zinc-induced stabilization of APC induces G2 cell cycle arrest in HCT116 cells that express full-length APC (127). β-catenin stabilization can also lead to G2/M cell cycle arrest (111). However, this arrest potentially reflects β-catenin's ability to sequester endogenous APC (128). Together, these studies suggest that APC participates in cell cycle regulation in a variety of cell types. The mechanism by which APC regulates G1/S cell cycle progression is presumably through suppressing Cyclin D1 in Wnt signaling. However, how does APC regulate G2/M cell cycle progression is less understood. Therefore, I focus on studying potential pathways through which APC controls G2-M cell cycle transition [see detail in Chapter 2&3].

Part III. Topoisomerase IIα

In an attempt to clarify the role of APC in cell cycle control, we identified a novel interaction between APC and topoisomerase IIα (topo IIα), a key regulator of the G2 decatenation checkpoint. Thus, I now introduce the basic biology of topo IIα protein.

Topo II enzyme and DNA topology

The intertwining feature of the DNA double helix (129) suggests that many biological process, such as DNA replication, are highly dependent on DNA topology (130). DNA is commonly under-wound or over-wound, making it difficult to separate the double helix to initiate replication, transcription, or other processes (131). In addition, knots and tangles accumulate during DNA recombination and replication. If not properly untangled during cell division, these knots will lead to cell death (132). Topoisomerases are enzymes that modulate DNA topology changes by creating transient breaks at phosphodiester bonds in the backbone of DNA double helices (133, 134). In eukaryotes, there are two types of topoisomerases with distinct catalytic mechanisms. Topoisomerase I (topo I) induces single-strand nicks in the double helix followed by single strand DNA passage to alleviate super-helical twists (135-137). Topo I is thus involved in most DNA processes to maintain genomic integrity (138). Topoisomerase II (topo II) generates double-strand breaks followed by a second double-strand DNA passage. Topo II is thought to be involved in more cellular activities than topo I (138-140).

Two human topo II genes have been identified: *TOP2α*, mapped to chromosome 17q21-22 (141); and *TOP2β*, mapped to chromosome 3q24 (142). The 170kDa topo IIα and the 180kDa topo IIβ are ~70% identical in amino acid sequence (143) and have similar primary structures. There are three major domains in topo II based on their

homology to bacterial and yeast topo II and proteolytic sites (Figure 1.5A) (136, 144, 145). The N-terminal domain (aa 1-~454) contains the ATP binding site (146). The central domain (aa ~454-~1263) contains the active tyrosine site that is required for DNA cleavage and re-ligation (138, 147). The C-terminal domain (aa ~1263-1521) is highly variable between two isoforms, thus may be responsible for the differences in the biological functions between topo IIα and β. This domain, although not required for topo II catalytic activity *in vitro*, contains the NLSs (148-150) and phosphorylation sites (151-153). The crystal structure of the catalytic domains in yeast topo II has been solved (Figure 1.5B) (144). The central domain of human topo IIα is homologous to the B-subunit and A-subunit of yeast topo II.

Topo IIα and topo IIβ function as homodimers to catalyze double-stranded DNA passage and facilitate changes in DNA topology (144, 154). A topo II dimer binds to two segments of DNA ---- the gated DNA helix and the transported DNA helix (Figure 1.6) (138-140, 144, 155). Upon ATP binding, the topo II dimer generates a double-strand break in the gated DNA helix, undergoes conformational change to form a closure that traps both segments in the central hole. This enzyme-cleaved DNA complex is called "cleavage complex". The nick in the gated DNA helix produces a "gate" to allow a second transported DNA helix to pass. Topo II then re-joins the DNA ends in the gated DNA helix and releases both DNA helixes by opening the dimer formed by B-subunits and C-terminal domains.

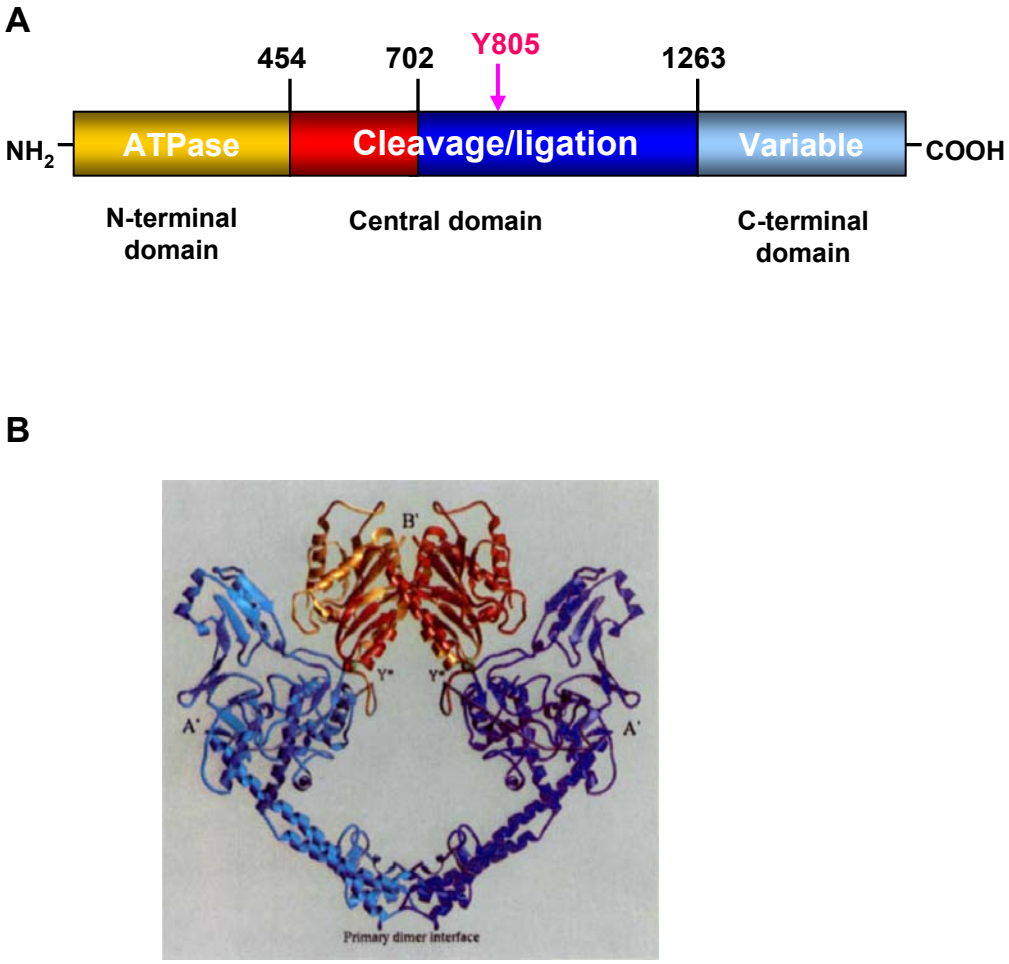

Figure 1.5

Structure of eukaryotic topo II protein. (A) The three domains of human topo IIα: N-terminal domain (yellow), Central domain (red and blue), and C-terminal domain (light blue), which is highly variable. Proteolytic cleavage sites are indicated at aa-454, aa-702 and aa-1263. The active site tyrosine residue Y805 is shown in pink. (B) Crystal structure of the catalytic active domain of yeast topo II (amino acid 401-1202). (Berger and Wang, 1996)

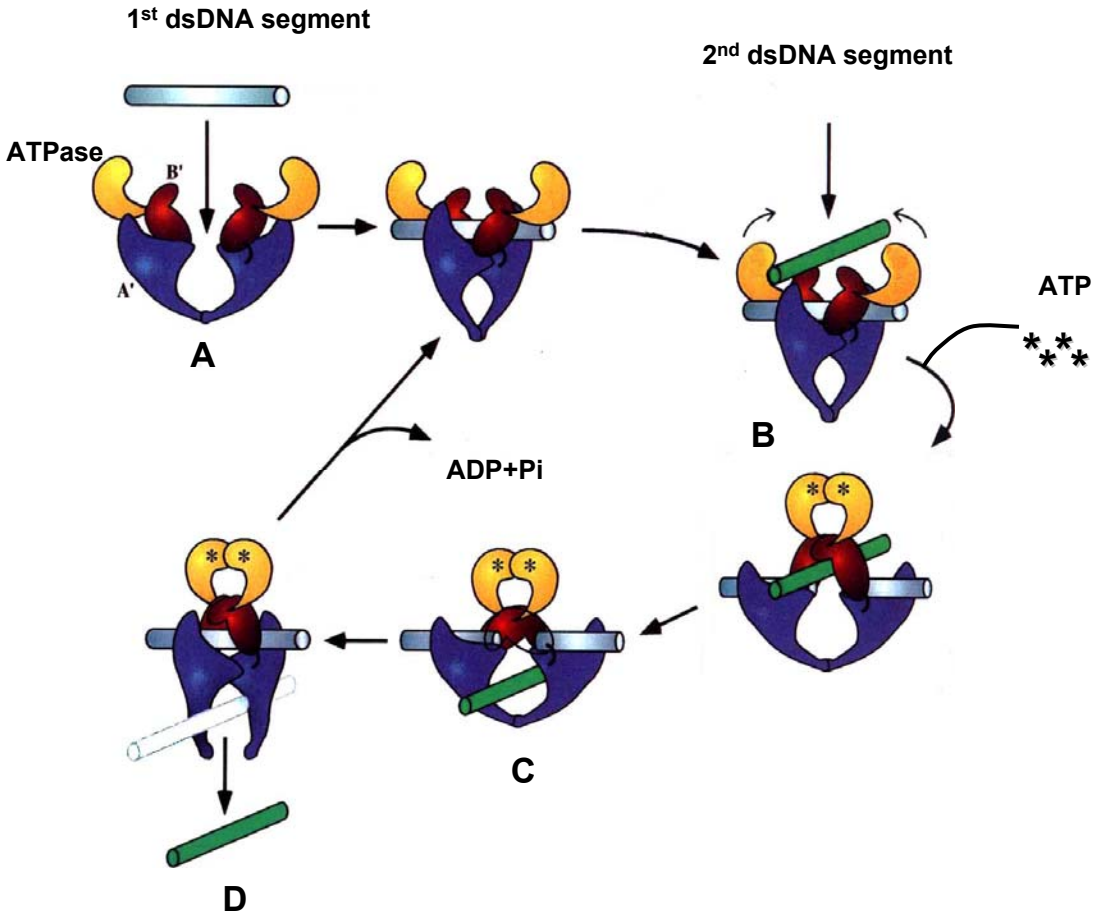

Figure 1.6

A molecular model of topo II catalytic action. DNA topo II is composed of three domains: ATPase domain (yellow), B' domain (red), and A' domain (blue). (A) The topo II dimer binds to the gated DNA helix (light blue). (B) The transported DNA helix (green) enters the complex between the ATPase domains. Meanwhile, ATP (stars) binds the complex to induce cleavage in the gated DNA helix and conformation change in the topo II dimer. (C) The transported DNA helix thus passes through the break in the gated DNA helix. (D) The intermediate "cleavage complex" is transient. rejoins the breaks in the gated DNA helix, and opens the closure to release the transported DNA helix. ATP is then hydrolyzed to prepare the enzyme for the next round of catalytic reaction. (Modified from Berger and Wang, 1996)

Topo IIα and the G2 decatenation checkpoint

Topo IIα and topo IIβ display similar enzymatic features, however, exhibit distinct expression patterns and physiological functions. Topo IIα expression fluctuates during cell cycle due to changes in mRNA stability (156, 157). Generally, topo IIα protein increases during cell proliferation, and progressively decreases when cells differentiate (143, 156). During cell cycle, topo IIα mRNA increases when cells progress from G0/G1 phase to S phase, reaching its maximum in G2 (158, 159). Topo IIα expression gradually declines toward the end of mitosis. In contrast, topo IIβ protein level and activity remain steady throughout the cell cycle (143, 156). Topo IIα accumulates at mitotic centromeres and during prophase of meiosis [reviewed in (145)]. The cell cycle-dependent expression pattern suggests a potential role for topo IIα in cell cycle control. The increasing level of topo IIα phosphorylation in G2/M of the cell cycle (158-160) also indicates a role for topo IIα in cell cycle regulation.

Mammalian cell lines lacking topo IIβ are viable in culture, suggesting that topo IIβ is dispensable for cell cycle progression, and topo IIα is sufficient for mitosis (161-163). Topo IIα has been shown to involve in chromosome condensation and segregation, although its precise role remains unclear. When treated with topo II poisons or inhibitors, cultured mammalian cell lines exhibit G2 arrest right before entering M phase (164-166). In most cases, topo II poisons trap DNA within the “cleavage-complex”. Therefore, the

cell cycle arrest observed resembles activation of the G2 checkpoint in response to DNA damage. However, in cases where topo II inhibitors, such as ICRF-193, do not induce formation of the stable "cleavage-complex", G2 cell cycle arrest still occurs (167-169). These results indicate that accumulation of incomplete decatenation of interlinked DNA due to topo II inhibitors or poisons halts cells at the G2 phase of the cell cycles thus activating the decatenation checkpoint. This G2 decatenation checkpoint delays mitotic entry until chromosomes are properly decatenated (168).

At present, our understanding of the G2 decatenation checkpoint is still limited. Until recently, only topo IIα was known to directly control the G2 decatenation checkpoint (170). The most popular current model, although lacking direct supporting evidence, is described in Figure 1.7 (171). The decatenation checkpoint is dependent on ATM- and Rad3-related kinase (ATR), which mediates the delay by inhibiting Polo-like kinase 1 (Plk1) (172). In normal cell cycle progression, Plk1 phosphorylates cyclin B1 to promote nuclear import of the cyclin B1/Cdk1 complex and thus execute cell cycle progression into mitosis (173). Treatment with topo II inhibitor ICRF-193 reduces Plk1 activity, thus inhibiting the G2-M transition by activating the decatenation checkpoint (173). Breast cancer 1 (BRCA1) and Werner helicase (WRN) are also required for the G2 decatenation checkpoint (172, 174). Mutation of either BRCA1 or WRN results in defects in the decatenation checkpoint. Restoration of a wild type BRCA1 or WRN reverses these defects.

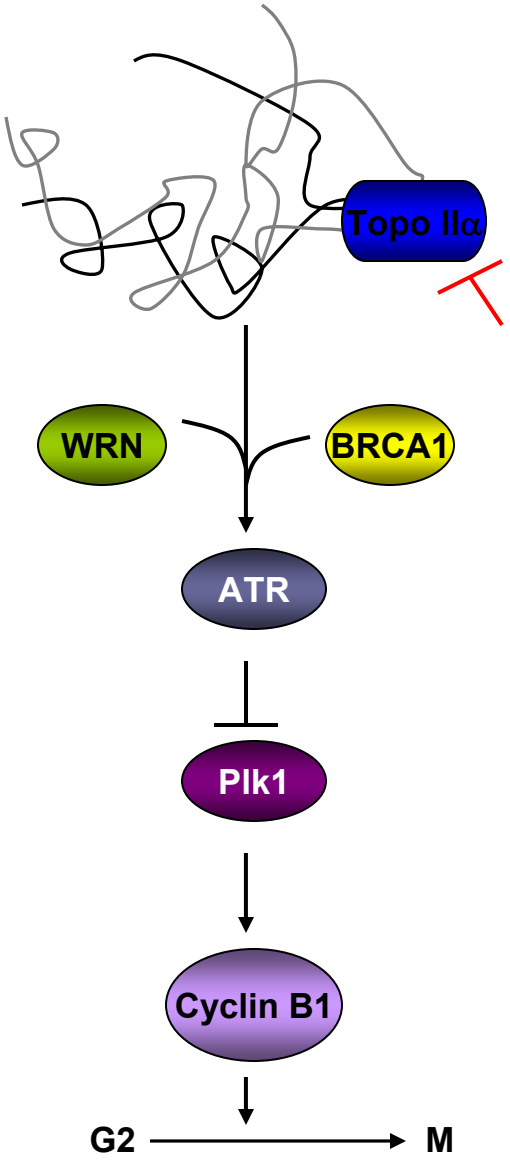

Figure 1.7

A molecular model of G2 decatenation checkpoint. Topo IIα is required to detangle chromosomes in G2 before entering M. If chromosome is not properly decatenated, ATR kinase can be activated in the presence of WRN and BRCA1. ATR kinase in turn inhibits Plk1, which is required for cyclin B1 phosphorylation and cyclin B1/Cdk1 nuclear localization. As a result, the decatenation checkpoint is activated and G2-M transition is delayed. (Modified from Damelin and Bestor, 2007)

Topo IIα also acts as a scaffold for p53 and Cdc2 (175). Cdc2 kinase association with chromatin is dependent on interaction with topo IIα. Inactivation of Cdc2 kinase is believed to induce G2 arrest (176). Therefore, inhibition of topo IIα activity may also perturb the G2-M transition through compromising Cdc2 activation. However, whether topo IIα is required for chromosome condensation is still controversial. One study reported that association between Cdc2 and topo IIα was required for chromatin condensation at the onset of mitosis using nuclear extract from chicken embryos (177). While others showed that in Hela cells with topo IIα efficiently knocked down using siRNA, chromosomes were properly condensed and mitotic spindles were assembled (178). These cells did not display any defects in G2-M transition either. These seemingly contradictory results, however, were obtained from two completely different systems. On the one hand, the *in vitro* chicken embryo nuclear extract may not function properly like a chicken embryo under normal physiological conditions. On the other hand, as transformed cancer cells, Hela cells may have escaped some important growth restrictions and thus might not have normal biological responses upon depletion of topo IIα protein. Further *in vivo* studies are required to reveal definitive biological functions of topo IIα in the G2-M transition and the mitotic regulation.

To better understand how topo IIα regulates cell cycle, identification of novel topo IIα binding partners that is involved in decatenation checkpoint is a practical approach. Such studies should be encouraged to explore the biological relevance of topo IIα

binding proteins. Thus, in Chapter 2&3, I introduced the finding of interaction between topo IIα and APC, the latter of which is also involved in G2/M cell cycle progression. Studying the functional interaction between these two proteins helps obtain mechanistic information of the topo IIα-dependent G2 decatenation checkpoint.

Topo IIα and CRC

Topo IIα expression is restricted to tissues containing many cycling cells, such as thymus, spleen, bone marrow, intestine and testis (179-181). In contrast, topo IIβ is detectable in a wider rage of tissues, but at a lower level than topo IIα, if both are present (180). In the normal colon, topo IIα expression is limited to the proliferative zone. However, topo IIα is up-regulated in many colon cancer tissues (182). At primary locations of recurrent malignant colon tumors following chemotherapy, the number of topo IIα-positive cells greatly increased compared to at primary sites with no recurrence (183). A recent study looking for biomarkers of CRC found *TOP2α* mRNA up-regulated four-fold in cancer compared to normal tissue (184).

Topoisomerases participate in almost all cellular functions involving DNA. Therefore, studies of topoisomerases have spanned both pharmacology and clinical medicine: from identification of antibiotics to target DNA gyrase in bacteria (185-187) to anticancer agents that target human topo I and II (188-191). Drugs targeting topo II have focused on topo IIα, because drug-resistant cell lines with eliminated topo IIβ have been

generated, suggesting cells lacking topo IIβ are viable (192). Most drugs are topo II poisons, targeting topo II by stabilizing the cleavage complex (193, 194). The resulting accumulation of double-stranded DNA breaks is toxic to cells. Other drugs inhibit topo II activity but do not stabilize the cleavage complex and some can prevent the transported DNA helix from passing through (195). The exact mechanisms of inhibition by these drugs that target topo II requires further investigation.

Although topo II poisons have been widely used as chemotherapeutic agents, cellular drug-resistance is a problem. Defects in the decatenation checkpoint have been observed in cultured normal stem cells, resulting in an increasing potential for genomic instability (196). Therefore, it has been proposed that cancer stem cells have similar defects, thus leading to tumor growth and progression by rapid generation of addition mutations (171). Further studies on cancer cell lines and tissues will add to our understanding of the importance of the decatenation checkpoint in tumorigenesis and tumor progression.

Part IV. Summary

The homeostasis of the intestinal epithelium is tightly controlled by complex synergism between multiple regulatory pathways. As discussed, no two CRCs are exactly alike and thus, their genesis can not be explained by a single oncogenic mechanism. Rather, CRCs result from the interplay of distinct mutations acting under different physiological conditions. Mutation of *APC* is clearly a key factor for colorectal

tumorigenesis. Loss of functional APC may affect intestinal epithelial homeostasis by altering a single pathway, such as Wnt signaling. However, extensive cross-talk between multiple functions of APC adds complexity to understanding the contribution of each APC activity. The combination of various effects makes APC a powerful tumor suppressor protein. Further study of some of the less understood APC functions, such as cell cycle regulation and cytoskeletal organization, will help elucidate how multiple pathways cooperate to prevent carcinogenesis. In the following chapters, we present evidence for a role of APC in the topo IIα-dependent G2-M transition and in intermediate filament regulation.

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CHAPTER 2

INTERACTION BETWEEN TUMOR SUPPRESSOR APC & TOPOISOMERASE IIα: IMPLICATION FOR THE G2/M TRANSITION

Abstract

The tumor suppressor APC is implicated in regulating multiple stages of the cell cycle. APC participation in G1/S is attributed to its recognized role in Wnt signaling. APC function in the G2/M transition is less well established. To identify novel protein partners of APC that regulate the G2/M transition, APC was immunoprecipitated from colon cell lysates and associated proteins were analyzed by MALDI-TOF. Topoisomerase IIα (topo IIα) was identified as a potential binding partner of APC. Topo IIα is a critical regulator of G2/M transition. Evidence supporting an interaction between endogenous APC and topo IIα was obtained by co-immunoprecipitation, colocalization, and FRET. The 15 amino acid repeat region of APC (M2-APC) interacted with topo IIα when expressed as a GFP-fusion protein *in vivo*. Although lacking defined nuclear localization sequences, M2-APC predominantly localized to the nucleus. Furthermore, cells expressing M2-APC displayed condensed or fragmented nuclei and were arrested in the G2 phase of the cell cycle. Although M2-APC contains a β-catenin binding domain, biochemical studies failed to implicate β-catenin in the observed phenotype. Finally,

purified recombinant M2-APC enhanced topo IIα activity *in vitro*. Together, these data support a novel role for APC in the G2/M transition, potentially through association with topo IIα.

Introduction

The tumor suppressor protein adenomatous polyposis coli (APC) is inactivated in over 80% of all colorectal cancers (1). The detection of mutant APC in the earliest stages of polyp development supports the idea that mutation of *APC* is an initiating event in colon carcinogenesis. The most common form of *APC* mutation results in elimination of the carboxy-terminal half of the APC protein. Because APC is a large, multi-domain protein, APC truncation is predicted to impact a number of cellular mechanisms, the extent of which we are only beginning to understand.

There is accumulating evidence supporting a role for APC in the regulation of the cell cycle. Over-expression of APC in NIH3T3 fibroblasts and colon cancer cell lines leads to G1 cell cycle arrest (2, 3), presumably by repressing transcription of Wnt targets such as cyclin D1. APC may also participate directly in mitosis as it is transiently hyperphosphorylated in the M phase of the cell cycle (4), accumulates at the microtubule organizing center (5) and associates with the kinetochore in dividing cells (6, 7). A role for APC in mitosis might be critical for regulation of genomic stability and proper

chromosome segregation. APC stabilized by zinc treatment induces G2/M cell cycle arrest in colon cancer cells (8). However, to date, little is known about the underlying mechanism by which APC participates in the G2/M cell cycle transition.

Here we report identification of topoisomerase IIα (topo IIα) as a potential APC binding protein. Topo IIα enzyme catalyzes DNA topology changes by introducing double strand DNA breaks that facilitate DNA strand passage and subsequent DNA re-ligation (9-12). Topo IIα has been implicated in a number of cellular functions such as DNA replication and chromosome condensation (9, 11-15), and appears essential in the control of the G2/M decatenation checkpoint during cell division (16). Topo IIα was also found to be deregulated in colon cancers with its expression limited to the proliferative zone in the normal colon, but up-regulated and widespread in colon cancer tissue (17). At primary locations of recurrent malignant colon tumors following chemotherapy, the number of topo IIα-positive cells is greatly increased compared to primary sites with no recurrence (18), suggesting that changes in topo IIα expression occur subsequent to *APC* mutation. Together, these observations make topo IIα an attractive candidate for mediating the G2/M cell cycle transition.

Over-expression of an APC fragment that interacts with topo IIα in various colon cancer cells lines led to abnormal nuclear morphology and cell cycle inhibition in G2.

Our data suggest a novel role for nuclear APC in the regulation of cell cycle progression, potentially through an interaction with topo IIα.

Materials and Methods

Cell culture and DNA constructs

HCT116βw cells (a generous gift from Dr. Bert Vogelstein) were grown in McCoy's 5A medium (Gibco) supplemented with 10% FBS (Hyclone). Expression constructs for APC fragments fused to GFP were kindly provided by Dr. Naoki Watanabe and have been described previously (19).

Antibodies and immunofluorescence microscopy

Immunostaining was performed as described (20) and the following antibodies were used: anti-APC (ab-7, 1:50, Calbiochem), anti-APC (ab-4, 1:2000, Calbiochem), anti-APC (ab-1, 1:100, Calbiochem), anti-topo IIα (1:100, Research Diagnostics, Inc.), anti-phospho-histone H3 (1:500, Upstate), anti-β-catenin (1:200, Transduction Lab) and anti-PCNA (1:1000, Transduction Lab); goat anti-mouse IgG Alexa 488 (1:1000, Molecular Probes), goat anti-rabbit IgG Alexa 568 (1:1000, Molecular Probes), and goat anti-mouse IgG Alexa 610-R-phycoerythrin (1:500, Molecular Probes). DNA was labeled with Topro-3 (1:500, Molecular Probes) or DAPI (1:5000, Invitrogen). 2-D and 3-D

distributions of immunofluorescent signals were examined using a Yokugawa-type spinning disk confocal microscope equipped with an Olympus 150X objective with a N.A. of 1.45 and 1KX1K EMCCD (Olympus and Intelligent Imaging Innovations, Denver, Co.). From over 200 cells that viewed, 50 cells were randomly chosen to be imaged. No less than 24 image frames were collected at z-intervals of 100 nm for image sets where 3-D co-localization was examined. No deconvolution was performed. All raw confocal image series were analyzed by ImageJ program and the JACoP plugin without further processing. Mitotic indices were determined by counting DAPI-stained mitotic cells in a field of 100 cells for each repetition.

Protein co-localization analysis

Colocalization coefficients were calculated using ImageJ and the JACoP plugin (21). Using Costes' method of automatic thresholding, a Pearson's coefficient was calculated for pixels within all of the calculated regions of interest in an image where Alexa 488 and Alexa 568 fluorescence were each detected at levels significantly above background. Mander's coefficients were also calculated in order to determine the degree of overlap between the corresponding regions of detected signals.

FRET analysis

Evidence of Förster resonance energy transfer (FRET) between secondary fluorophores was detected using a Zeiss 510 Meta spectral imaging upright laser scanning confocal microscope. Donor fluorescence, alternatively from Alexa 488 or Alexa 610-R-phycoerythrin was photo-bleached by passing the beam of the 543 nm laser (at 100% output) over defined regions of interest (ROI) for 75 iterations or using 200 passes of the 633 nm laser (also 100% output). Close proximity of the two proteins was determined by calculating the mean FRET efficiency between donor and acceptor according to the method of Kenworthy and Edidin (22) where E=100(Alexa 488_{post}-Alexa 488_{pre})/Alexa488_{post} or E=100(Alexa 610-R-PE_{post}-Alexa 610-R-PE_{pre})/Alexa 610-R-PE_{post}. All pixels within the photo-bleached regions were used for these calculations. Error bars indicate the Standard Error of the Mean (SEM). Three dimensional reconstructions and projections were also performed with Image J.

Immunoprecipitation and western immuno blots

Immunoprecipitation (IP) and western immuno blots (IB) were performed using modified standard protocols. Cells at 90% confluency were lysed in lysis buffer [50 mM Tris pH7.5, 0.1% NP40, 100 mM NaCl, 1 mM MgCl2, 5 mM EDTA, protease inhibitor cocktail (Sigma), and Halt phosphatase inhibitor cocktail (PIERCE)] on ice for 30

minutes. Cell lysates were sonicated for 10 pulses, level 1 with 10% output, 3 times. Specific antibodies were pre-incubated with Protein A dynabeads (Invitrogen) for 2 hours at room temperature. Dynabeads saturated with antibodies were added to 1mg of soluble lysate and incubated overnight at 4°C. IP pellets were subject to two washes of 15 minutes using lysis buffer and one wash using PBS-T at 4°C. The following antibodies were used for IP: anti-topo IIα sera (a generous gift from Dr. Joe Holden), affinity purified anti-APC-M2 rabbit polyclonal antibody made against amino acid 1000-1326, and polyclonal anti-GFP (Invitrogen). Immunoblots were probed with the following antibodies: anti-APC (ab-1, 1:100, Calbiochem), anti-APC-M2 polyclonal (1:4000), anti-β-catenin (1:2000, Sigma), anti-topo IIα (1:1000, Research Diagnostics, Inc.), anti-topo IIβ (1:1000, Santa Cruz) and anti-α-tubulin (1:2000, Oncogene).

Transfection and reporter gene assay

HCT116βw, SW480 and HCA7 cells were transfected using Lipofectamine2000 reagent according to the manufacturer's protocol (Invitrogen). For luciferase assays, HCT116βw cells grown in 24-well plate were cotransfected with 2 μg of the GFP-M2-APC or GFP expression construct, 100 ng of the TCF-reporter construct SuperTOP-flash or FOPflash (Upstate Biotechnology, Lake Placid, NY), and 50 ng of the pRL-TK *Renilla* luciferase construct (Promega, WI) as a control to normalize the transfection efficiency. After 24 hrs, cells were harvested and luciferase activities were

determined using Dual-Luciferase® assay system (Promega) and a Turner Designs TD-20/20 luminometer. SuperTOP-flash and FOPflash luciferase activities were first normalized by pRL-TK *Renilla* luciferase, and then the normalized SuperTOP-flash luciferase activity was divided by normalized FOPflash luciferase activity to calculate relative β-catenin activity.

FACScan analysis

Propidium iodide staining of GFP expressing cells in suspension was performed using a standard protocol as described (23). HCT116βw cells at 35% confluency were transfected using Lipofectamine 2000 (Invitrogen). 30 hours post transfection, cells were fixed with 2% paraformaldehyde (Electron Microscopy Science) on ice for 1 hour, followed by overnight permeabilization using 70% ethanol in PBS. Rehydrated cells were then stained with 40 μg/ml propidium iodide (Sigma) in PBS for 30 minutes at 37°C. FACS analysis was performed using a Beckton Dickinson FACScan.

Topo IIα assays and recombinant proteins

To generate recombinant S tag fused M2-APC, the corresponding cDNA for APC (amino acid 1000-1326) was subcloned into a pET-30a(+) vector. Both tags (S and His) were fused to the amino terminus of the protein. The expression and purification of S-M2-APC fusion protein was performed as described (24). Recombinant human topo

IIα was made as described (25, 26). *In vitro* topo IIα relaxation and decatenation assays were performed as described (10).

Results

Endogenous full-length APC associates with topo IIα

To identify novel APC binding proteins that could potentially function to regulate cell cycle progression, we immunoprecipitated APC from HCT116 cell lysates, resolved precipitated proteins by SDS-PAGE, and visualized these proteins using coomassie blue. One protein in a 170 kDa band unique to APC precipitation was identified by MALDI-TOF analysis as topoisomerase IIα (topo IIα) [Neufeld & White, unpublished].

We verified the APC / topo IIα interaction in both HCT116 cells and cells derived from this cell line. Although HCT116 cells were initially cultured from human colon cancer tissue, they express full length APC and maintain a stable karyotype. The original HCT116 cell line possesses one wild-type and one mutant β-catenin allele. The mutant allele encodes a stabilized version of β-catenin which is not down-regulated by APC. The HCT116βw line we use for most experiments was engineered to eliminate the mutant β-catenin allele and thus, expresses only wild-type β–catenin protein (27). Full-length endogenous APC co-immunoprecipitated with topo IIα from HCT116 cell lysates (Figure 2.1A). No full-length APC was precipitated with control rabbit IgG. In

reciprocal experiments, topo IIα co-precipitated with full-length APC using an affinity purified APC antibody, but not with preimmune sera (Figure 2.1B, left panel). We consistently detected nearly 2% of the total topo IIα co-precipitated with APC (Figure 2.1B, right panel). In contrast, although topoisomerase IIβ is 75% identical to topo IIα, topo IIβ did not co-precipitate with APC (Figure 2.1B, left panel). This apparent binding preference for topo IIα over topo IIβ increases the likelihood that the APC topo IIα interaction is specific.

The subcellular distribution of endogenous APC and topo IIα further implicated topo IIα as a binding partner of APC. Endogenous APC was found to colocalize with topo IIα within single confocal slices taken through the nucleus where both appear as overlapping puncta (Figure 2.1C, a). When the entire cell thickness was visualized by a series of confocal images captured in the z-plane, areas of overlap were apparent throughout the nuclei (Figure 2.1C, b). To show the areas of overlap from an entire cell thickness as a single image, overlapping signals in the 3-D data sets were projected onto a 2-D surface and are displayed in yellow (Figure 2.1C, c). The degree of overlap between the corresponding regions was calculated for each 2-D confocal section and is displayed as the average for the entire imaged volume. On average, 1.9% of the topo IIα signal coincided with APC, and 10% of the APC signal coincided with topo IIα (Figure 2.1C,d).

To further examine the association between APC and topo IIα, we analyzed Förster resonance energy transfer (FRET) between the two immuno-labeled proteins in fixed, permeabilized HCT116βw cells (Figure 2.1D). Given the nature of FRET, it is estimated that energy transfer would be detected only if two antibody-labeled proteins are less than 30 nm apart. Detection of an average energy transfer efficiency (E) of 20.8% between immunolabeled Alexa 488-APC and Alexa 568-topo IIα within photobleached regions of interest indicates that endogenous APC and topo IIα are in close proximity and is consistent with a direct interaction in colon epithelial cells. This value is significantly more positive than measurements performed on cells in which photobleaching was omitted (E=-0.9%). Likewise, cells transiently expressing GFP showed no energy transfer between endogenous topo IIα and exogenous GFP (E=-5.7%), even though GFP was abundant in the nucleus (see e.g. Figure 2.5A).

The 15-amino acid repeat region of APC colocalizes with topo IIα, alters nuclear morphology, and causes cell cycle arrest in G2

APC is a 310 kDa protein with several distinct protein-binding domains (Figure 2.2A). To identify potential topo IIα-binding domains in APC, we expressed five GFP-fused APC fragments (Figure 2.2A) in HCT116βw cells and compared their localizations to that of endogenous topo IIα (Figure 2.2B). As expected, GFP fused with either NT-APC, M1-APC or CT-APC predominantly localized to the cytoplasm (Figure

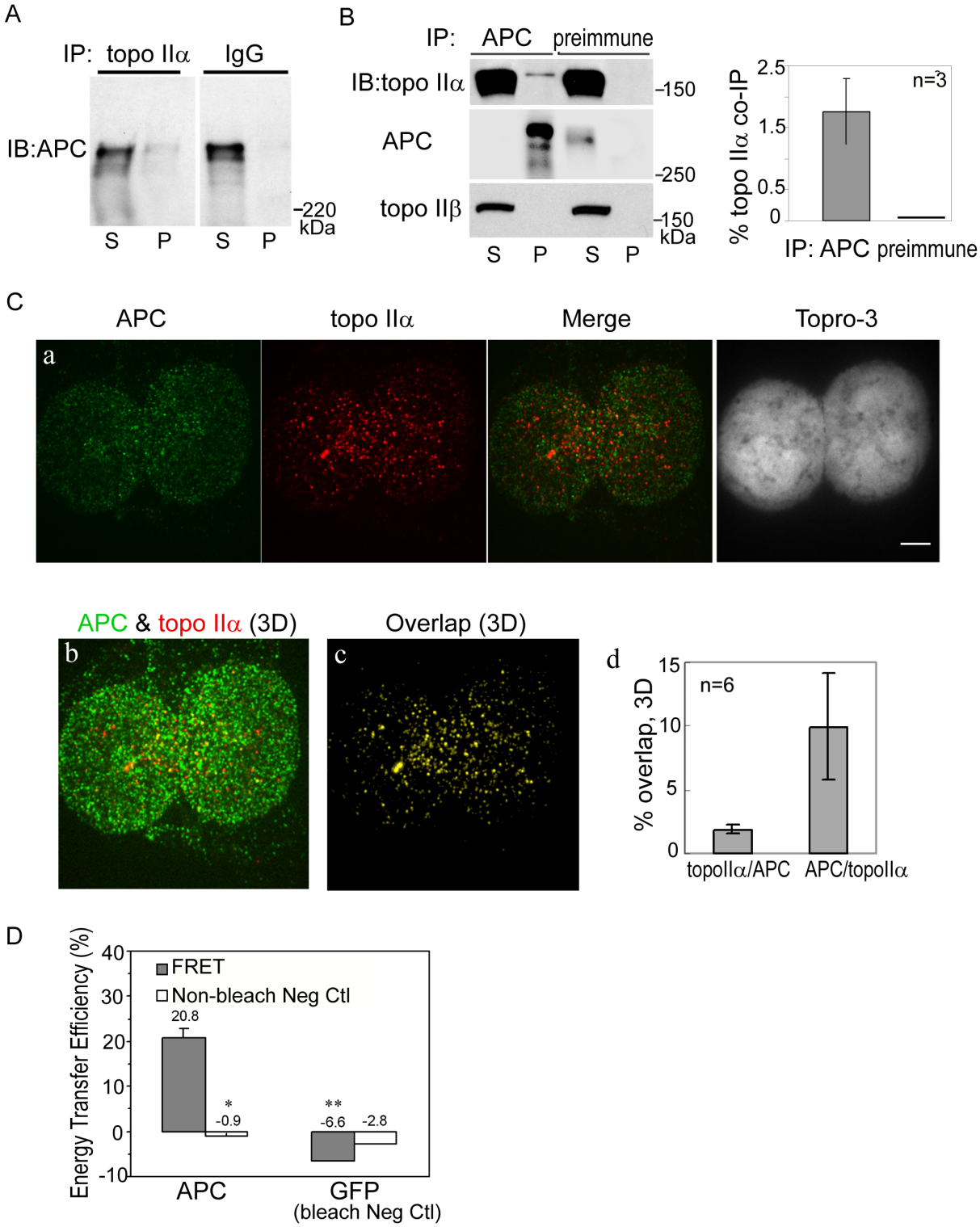
A
IP: topo IIα
IgG
IB:APC
220 kDa
S P S P
B
IP: APC preimmune
IB:topo IIα
APC
topo IIβ
150
250
150
kDa
S P S P
% topo IIα co-IP
2.5
1.5
0.5
0
n=3
IP: APC preimmune
C
APC
topo IIα
Merge
Topro-3
a
APC & topo IIα (3D)
Overlap (3D)
b
c
d
% overlap, 3D
15
10
5
0
n=6
topoIIα/APC
APC/topoIIα
D
Energy Transfer Efficiency (%)
40
30
20
10
0
-10
FRET
Non-bleach Neg Ctl
20.8
*
-0.9
**
-6.6
-2.8
APC
GFP
(bleach Neg Ctl)

Figure 2.l

Endogenous topo IIα associates with endogenous full-length APC. (A) Endogenous full-length APC co-immunoprecipitated with topo IIα using anti-topo IIα rabbit sera, but not using rabbit IgG. P, precipitated proteins; S, nonprecipitated supernatant proteins. Representative blot from eight independent experiments. (B) Full length APC immunoprecipitated with affinity purified polyclonal anti-APC sera (left, middle panel). Endogenous topo IIα (upper panel) co-immunoprecipitated with the endogenous full-length APC while topo IIβ did not (lower panel). Quantification of the band intensity from 3 independent experiments revealed 1.8% of the total topo IIα co-precipitated with full-length APC (right panel). Representative blots from seven independent experiments. (C) Colocalization of endogenous APC and topo IIα in HCT 116βw cells using APC antibody (ab-7), polyclonal topo IIα antibody and confocal microscopy. (a) Confocal image shows APC (green) and topo IIα (red) colocalized in the nucleus (yellow in Merge). Bar, 5 μm. (b) Confocal images through the entire cell thickness were collected for APC (green) and topo IIα (red) and are shown as a projection of the 3D Z-series images. (c) Overlapping pixels for the Z-series images in b are projected and shown in yellow. (d) Graph shows the average number of overlapping pixels calculated from six individual Z-series images. 1.9% of the topo IIα pixels overlap with APC, while 10% of the APC pixels overlap with topo IIα. (D) Measurements of Förster resonance energy transfer (FRET) between APC-Alexa 488 and topo IIα–Alexa 568 were performed using the method of donor fluorescence sensitization following acceptor photo-bleaching in fixed samples of immunofluorescently labeled HCT116βw cells. Endogenous APC was labeled using either anti-APC ab-1 or anti-APC ab-7 followed by goat anti-mouse Alexa 488 secondary antibody. Endogenous topo IIα was labeled using anti-topo IIα antibody followed by goat anti-rabbit Alexa 568 secondary antibody. Energy Transfer Efficiencies (E) between immunolabeled Alexa 488-APC and Alexa 568-topo IIα were ~20.8% (grey bar, left, n=10) within photobleached regions. This value is significantly positive (* p = 0.00021) compared to E measured without photobleaching (E=-0.9%, n=10, white bar) or to energy transfer (E=-5.7%, n=3, grey bar, right) between photobleached GFP which is abundantly expressed in the nucleus and immunolabeled Alexa 488-APC (** p=0.0000000056).

2.2B). M3-APC (amino acids 1211-2075) contains all 20-amino acid repeats and two nuclear localization signals (NLS) (28) and appeared in the nucleus when fused to GFP. GFP fused with M2-APC (amino acids 959-1338) showed the most prominent overlap with topo IIα in the nucleus. This region of APC contains all four 15 amino acid β-catenin binding repeats and one 20 amino acid repeat but no defined NLS.

Not only was GFP-M2-APC predominantly localized to the nucleus, but HCT116βw cells expressing this APC fragment displayed abnormal DNA morphology, with nuclei appearing condensed or fragmented (Figure 2.3A). To quantify this phenotype, GFP-positive cells were scored at 24, 48 and 72 hours post transfection (Figure 2.3B and Table 2.1). GFP-M2-APC expression resulted in a dramatic alteration in the DNA morphology, with 82% of the cells displaying abnormal nuclei by 48 hours post transfection. In contrast, expression of GFP-M3-APC or GFP alone had little effect on DNA morphology. Because the abnormal nuclear morphology was not prevalent in M3-APC- or GFP-expressing cells, but rather was limited to cells expressing M2-APC, this phenotype is not merely the cellular response to over-expression of a nuclear protein. Moreover, abnormal nuclei were also observed in SW480, HCA7 (Figure 2.3C, Table 2.2) and parental HCT116 cells (data not shown) expressing GFP-M2-APC. Therefore, the abnormal nuclear morphology is not restricted to the HCT116βw cell line and is not dependent on full length endogenous APC. The abnormal nuclear morphology resulting

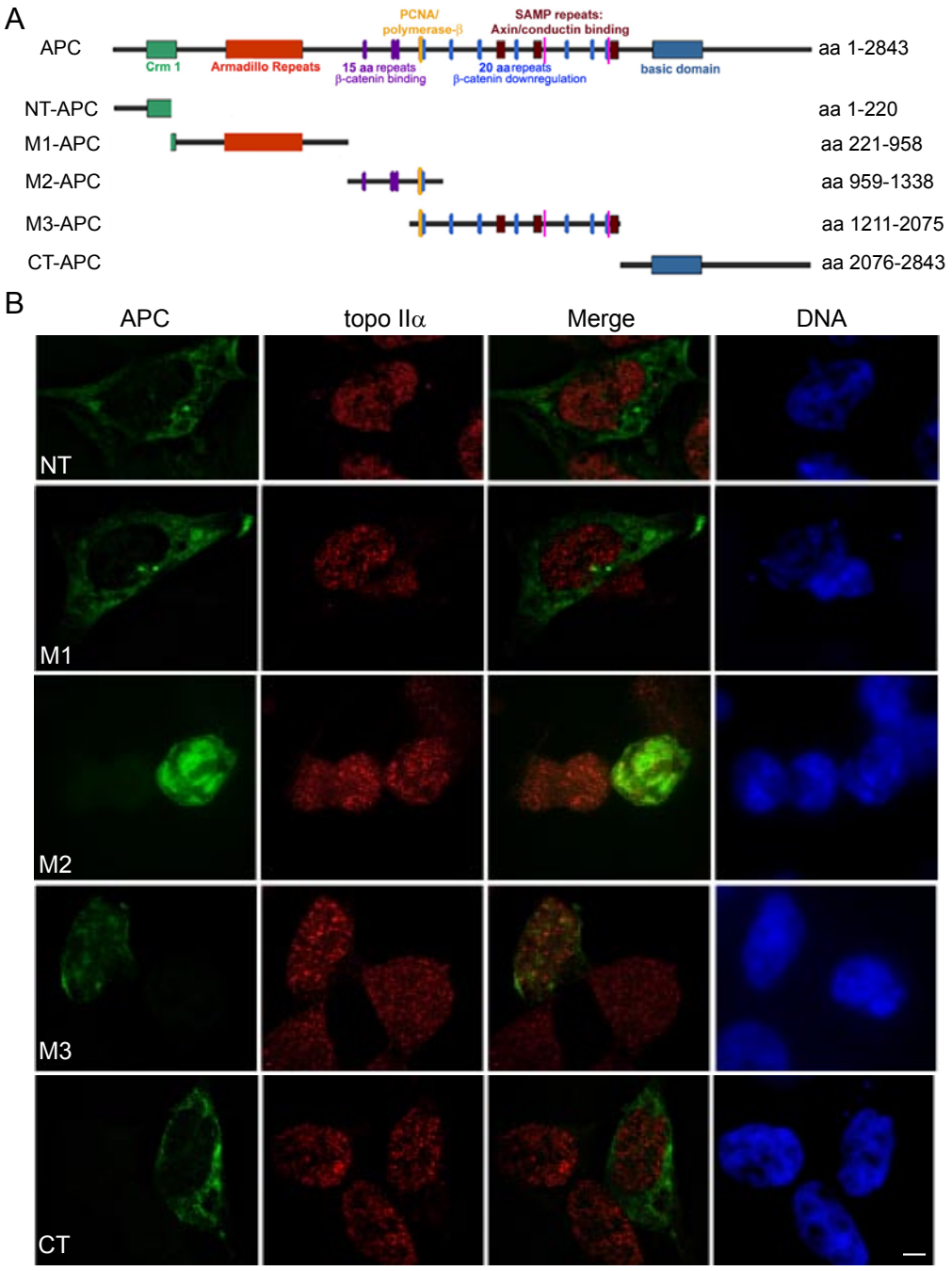

Figure 2.2
The 15-amino acid and 20-amino acid repeat regions of APC each colocalize with topo IIα and localize to the nucleus. (A) Schematic diagram of APC with domains implicated in nuclear function marked. Two NLSs are designated by thin pink lines in both full-length APC and M3-APC. Five APC fragments expressed as GFP fusions are shown. (B) Colocalization of the GFP-fused APC fragments with endogenous topo IIα in HCT116βw cells. Of the five APC fragments, both M2 and M3 displayed significant nuclear localization and partial colocalization with topo IIα. Bar, 5 μm.

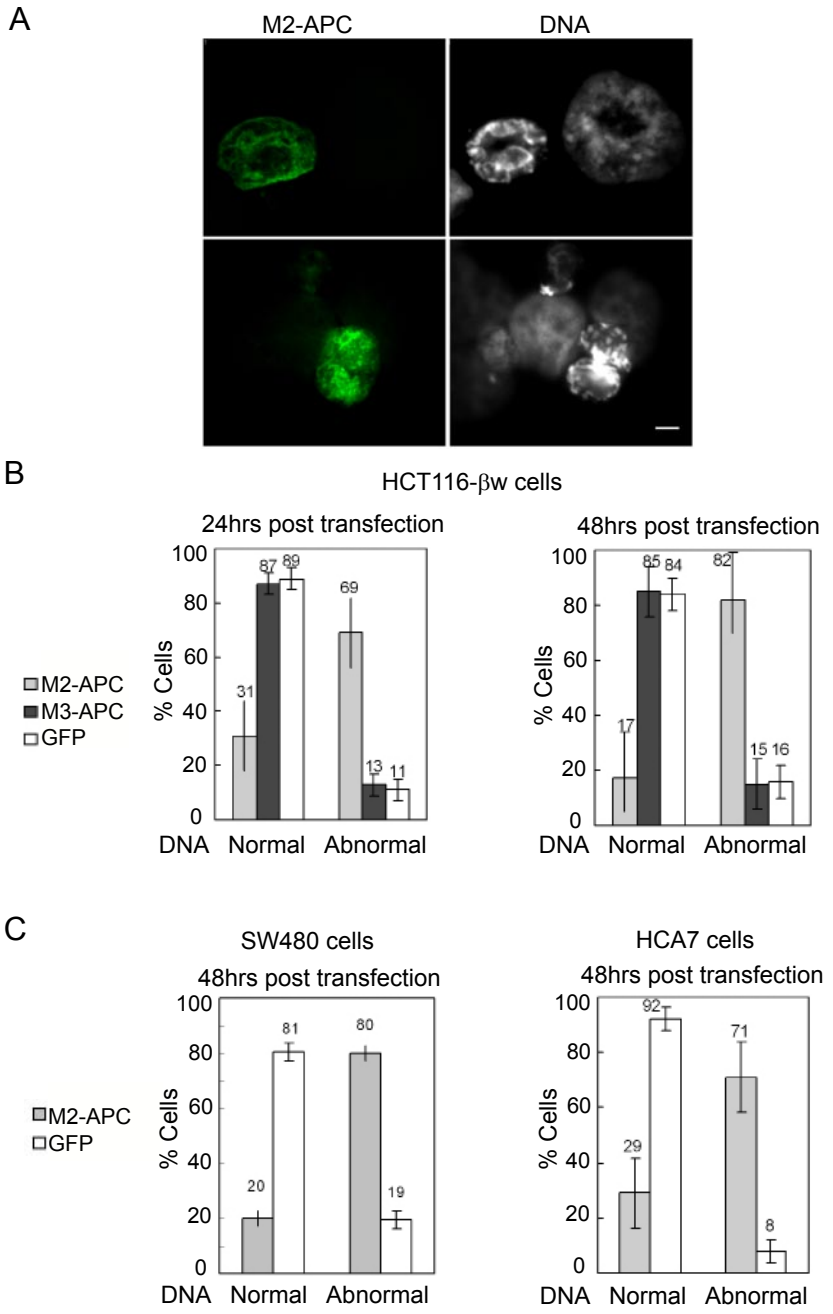

Figure 2.3
The 15-amino acid repeat region of APC protein alters nuclear morphology. (A) Confocal immunofluorescence microscopy revealed abnormal nuclei (condensed or fragmented) in M2-APC expressing HCT116βw cells. Bar, 5 μm. (B) Nuclear phenotype of HCT116βw cells expressing GFP-M2-APC (light bar), GFP-M3-APC (dark bar), or GFP (white bar) at 24 and 48 hours post transfection. Over 80% of the M2-APC expressing cells displayed abnormal nuclear morphology by 48 hours. (C) Nuclear phenotype of SW480 cells and HCA7 cells expressing GFP-M2-APC (light bar) or GFP (white bar) at 48 hours post transfection. (B, C) Graphs represent analysis of 100 cells for each transfection in three independent experiments with error bars indicating standard deviation.

Table 2.1 Nuclear phenotypes of HCT116βw cells expressing GFP, GFP-M2-APC or GFP-M3-APC

		Normal DNA	Abnormal DNA
24 hrs	GFP	89 +/- 4	11 +/- 2
	M2-GFP	31 +/-13	69 +/-13
	M3-GFP	87 +/- 4	13 +/- 4
48 hrs	GFP	84 +/- 6	16 +/- 6
	M2-GFP	17 +/- 11	82 +/- 12
	M3-GFP	85 +/- 9	15 +/- 9
72 hrs	GFP	89 +/- 7	11 +/- 7
	M2-GFP	7 +/- 3	93 +/- 3
	M3-GFP	65 +/- 27	35 +/- 27

For each transfection, 100 cells were chosen randomly and were scored in 3 independent experiments at three time points.

Table 2.2 Nuclear phenotypes of SW480 and HCA7 cells expressing GFP, or GFP-M2-APC

		SW480		HCA7	
		Normal. DNA	Abnormal. DNA	Normal DNA	Abnormal DNA
24 hrs	GFP	75 +/- 5	25 +/- 5	82 +/- 5	19 +/- 5
	M2-GFP	18 +/-3	82 +/-3	41 +/-6	59 +/-6
48 hrs	GFP	81 +/- 5	19 +/- 5	92 +/- 4	8 +/- 4
	M2-GFP	20 +/-3	80 +/-3	29 +/-13	71 +/-13
72 hrs	GFP	79 +/- 7	21 +/- 7	82 +/- 14	17 +/- 16
	M2-GFP	17 +/-4	83 +/-4	45 +/-7	55 +/-7

For each transfection, 100 cells were chosen randomly and were scored in 3 independent experiments at three time points.

from M2-APC expression resembled that seen in apoptotic cells. To determine if over-expression of M2-APC triggered apoptosis, we examined cells for various apoptotic markers such as activated caspase-3 and annexin V, but found no evidence of apoptosis (data not shown). Furthermore, when the cell cycle distribution of propidium iodide-stained cells was analyzed using FACScan, the GFP-M2-APC expressing cells had no detectable sub-G0 cell population indicative of apoptotic cells (Figure 2.4A). Overall, the cell cycle distribution of non-transfected cells was only slightly different from that of GFP-expressing cells. In contrast, the GFP-M2-APC-expressing cells showed a reproducible 2.5-fold increase in the G2/M phase and an accompanying reduction in the S phase compared to control cells (Figure 2.4B).

To determine whether this expanded G2/M population reflected an arrest in the G2 or M phase of the cell cycle, mitotic indices and phospho-histone H3 expression were both evaluated at 24, 48 and 72 hrs post transfection (Figure 2.4, C and D). None of the M2-APC-expressing cells were mitotic at any time point, whereas control cells or cells expressing GFP alone showed typical mitotic indices at all time points examined. These data suggest that the expression of M2-APC results in G2 cell cycle arrest.

The abnormal nuclear morphology following expression of the 15-amino acid repeat of APC is not due to altered β-catenin

M2-APC comprises all four of the 15 amino acid β-catenin binding repeats and one of the 20 amino acid repeats involved in β-catenin down-regulation (Figure 2.2A). Activated Wnt signaling resulting from stabilized β-catenin was recently reported to contribute to chromosome instability (29) and lead to G2 arrest (5). Thus, we predicted that the abnormal nuclear morphology seen in cells expressing M2-APC was dependent on M2-APC association with and stabilization of nuclear β-catenin. We found β-catenin expression and localization identical in HCT116βw cells expressing M2-APC, GFP, or non-transfected (Figure 2.5A). Furthermore, immunoblots from total lysates demonstrated comparable levels of β-catenin in M2-APC- and GFP-expressing cells (Figure 2.5D). Moreover, less than 1% of the total β-catenin co-precipitated with GFP-M2-APC (Figure 2.5B). In contrast, more than 10% of the total β-catenin was precipitated along with endogenous full-length APC in parallel experiments under the same experimental conditions (Figure 2.5C). Finally, β-catenin activity measured in M2-APC-expressing cells was comparable to that in GFP-expressing cells (Figure 2.5E). Thus, it appears unlikely that the abnormal nuclear morphology and the G2 cell cycle arrest observed in M2-APC-expressing cells result from β-catenin sequestration by M2-APC.

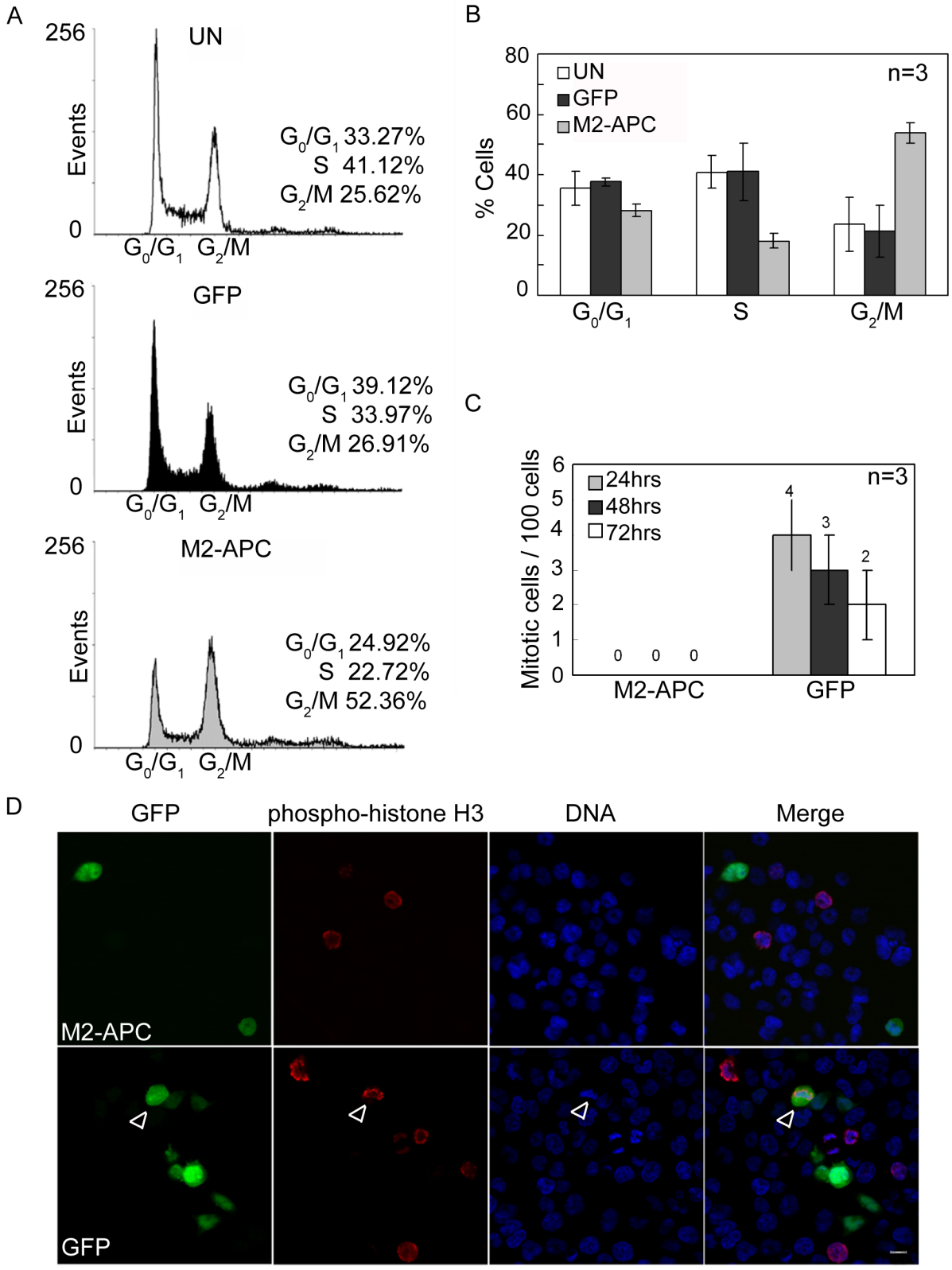
A
256
UN
Events
0
G0/G1 33.27%
S 41.12%
G2/M 25.62%
G0/G1 G2/M
256
GFP
Events
0
G0/G1 39.12%
S 33.97%
G2/M 26.91%
G0/G1 G2/M
256
M2-APC
Events
0
G0/G1 24.92%
S 22.72%
G2/M 52.36%
G0/G1 G2/M
B
80
60
40
20
0
% Cells
UN
GFP
M2-APC
n=3
G0/G1
S
G2/M
C
Mitotic cells / 100 cells
6
5
4
3
2
1
0
24hrs
48hrs
72hrs
n=3
4
3
2
0 0 0
M2-APC
GFP
D
GFP
phospho-histone H3
DNA
Merge
M2-APC
GFP

Figure 2.4

Expression of the 15-amino acid repeat region of APC results in G2 accumulation. (A) Histograms showing representative FACScan displays of cell cycle distribution assessed by propidium iodide staining. UN, untransfected cells. For both GFP and GFP-M2-APC expressing cells, only GFP positive cells are displayed. (B) FACScan data from three independent experiments. The fraction of cells in G2/M doubled, and the S phase decreased by half for cells expressing GFP-M2-APC compared to the untransfected control cells or cells expressing only GFP. Values for G_0/G_1, S, and G_2/M, respectively are: 35.6% +/- 2.1, 40.9% +/- 2.4, 23.5% +/- 3.3 (for Untransfected); 37.7% +/- 1.3, 41.0% +/- 9.6, 21.3% +/- 8.5 (for GFP); and 28.0% +/- 5.6, 18.1% +/- 5.6, 53.9% +/- 8.9 (for M2-APC). For each transfection, 15,000 GFP-positive cells were analyzed. (C) Mitotic events assessed following DAPI staining. One hundred randomly chosen M2-APC- or GFP-expressing cells were analyzed from three independent experiments at 24, 48 and 72 hours post transfection. None of the M2-APC-expressing cells appeared mitotic. A small number of the GFP-expressing cells were mitotic. (D) Representative immunofluorescence confocal microscopy of mitotic marker phospho-histone H3 from three independent experiments. No M2-APC-expressing cells were positive for phospho-histone H3, whereas a few GFP-expressing cells were positive. Arrow head indicates a cell that is positive for both GFP and phospho-histone H3. Bar, 10 μm.

The 15-amino acid repeat region of APC interacts with topo IIα

Our original identification of APC in complex with topo IIα, an essential regulator of the G2/M transition prompted us to examine whether M2-APC could interact with topo IIα and possibly mediate the G2 arrest through this interaction. HCT116βw cells transfection and normalized against both pRL-TK *Renilla* activity and FOP-flash reporter activity. Values are mean ±s.d. for triplicate samples from a representative experiment. transiently expressing M2-APC had increased levels of total topo IIα (Figure 2.6A) and the topo IIα co-precipitated with GFP-M2-APC (Figure 2.6B). In contrast, no topo IIα precipitated with the GFP in cells expressing GFP alone. Because M2-APC and topo IIα interact, it is possible that M2-APC can influence the activity of topo IIα. To test this directly, *in vitro* DNA relaxation and decatenation assays were performed using purified recombinant proteins topo IIα and M2-APC. Purified M2-APC stimulated topo IIα activity in both DNA relaxation (Figure 2.6 C) and decatenation assays (data not shown). This enhancement was apparent when purified M2-APC was provided at equal molar concentration to the topo IIα and was further pronounced with four times more M2-APC in the reaction (Figure 2.6C). In the absence of topo IIα, purified M2-APC did not relax the DNA (Figure 2.6C) or bind to the DNA slowing its migration (data not shown). Using a higher concentration of recombinant topo IIα, M2-APC still enhanced topo IIα activity (Figure 2.6D). In contrast, BSA protein at an equal molar ratio slightly

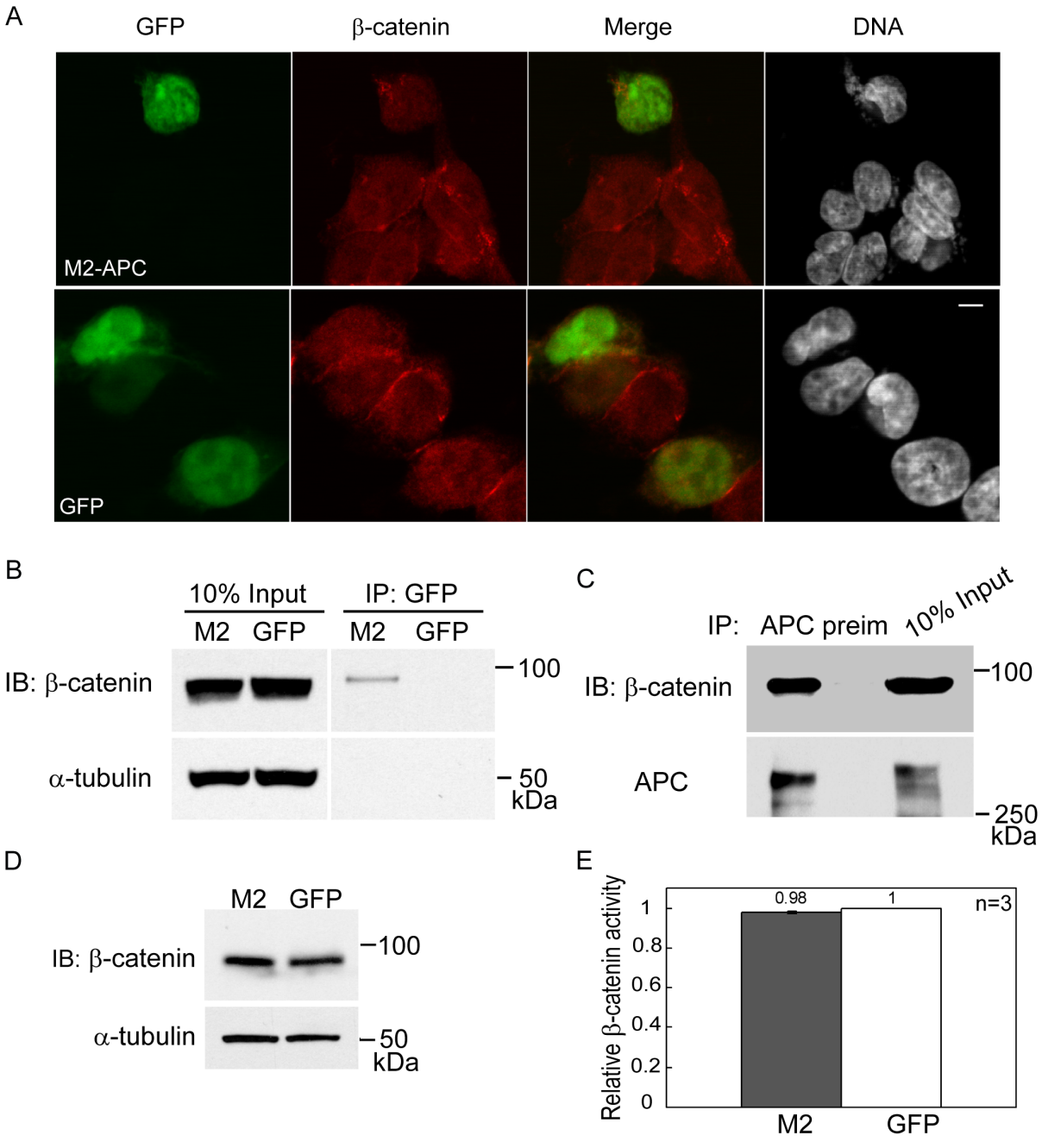
A
GFP
β-catenin
Merge
DNA
M2-APC
GFP
B
10% Input
IP: GFP
M2
GFP
M2
GFP
IB: β-catenin
α-tubulin
100
50
kDa
C
IP:
APC preim
10% Input
IB: β-catenin
APC
100
250
kDa
D
M2
GFP
IB: β-catenin
α-tubulin
100
50
kDa
E
Relative β-catenin activity
0.98
1
n=3
1
0.8
0.6
0.4
0.2
0
M2
GFP

Figure 2.5

Expression of the 15-amino acid repeat region of APC does not alter β-catenin expression or localization**.** (A) Confocal immunofluorescence microscopy reveals similar β-catenin localization in M2-APC- and GFP-expressing HCT116βw cells. Note, nucleus (right panel) is abnormal in M2-APC-expressing cell. Bar, 5 μm. (B) Less than 1% of the total β-catenin co-immunoprecipitated with GFP-M2-APC using a GFP antibody (M2) and none co-immunoprecipitated with GFP alone (GFP). 10% input is 25 μg total protein. Transfection efficiency was ~50%. (C) Nearly 10% of the total β-catenin co-immunoprecipitated with endogenous APC. 10% input is 30 μg total protein. Representative blots from five independent experiments. (D) Western blot reveals comparable levels of total β-catenin in M2-APC- (M2) and GFP-expressing (GFP) cells. Equivalent amounts of total protein from whole cell lysates were loaded. (B, D) Representative blots from three independent experiments. (E) Cells were cotransfected with GFP-M2-APC or GFP, SuperTOP-flash or FOP-flash reporters, and pRL-TK *Renilla* luciferase plasmid. Luciferase activities were determined 24 hours post transfection and normalized against both pRK-TK *Renilla* activity and FOP-flash reporter activity. Values are mean ±s.d. for triplicate samples from a representative experiment.

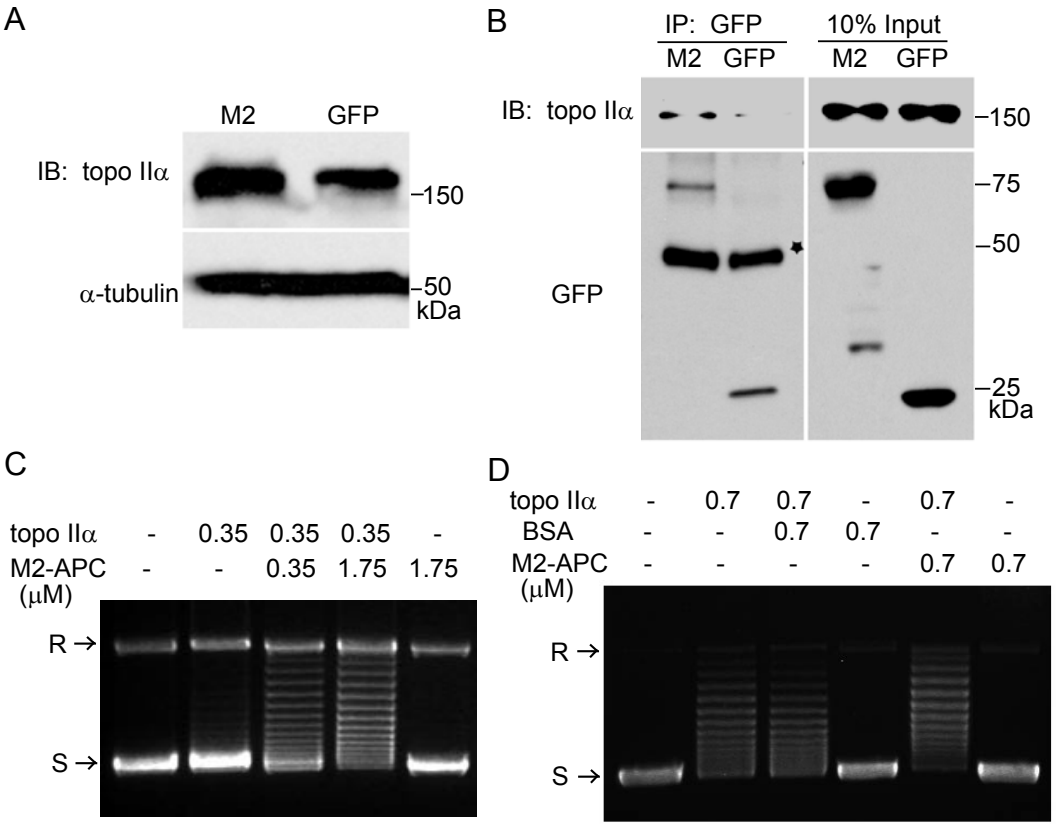

Figure 2.6
The 15-amino acid repeat region of APC associates with topo IIα and enhances topo IIα activity. (A) Increased expression of topo IIα in M2-APC-expressing cells compared to GFP-expressing cells. Equivalent total protein loaded in each lane. (B) Topo IIα co-immunoprecipitates with M2-APC, but not with GFP. 10% input is 25 μg total protein. (*) marks migration of the antibody heavy chain. (A, B) Representative blots from five independent experiments. (C, D) Representative topo IIα DNA relaxation assays from five independent experiments. R, relaxed plasmid DNA; S, supercoiled plasmid.. (C) Purified recombinant human topo IIα (0.35 μM) slightly relaxed supercoiled plasmid DNA (lane 2). Addition of purified recombinant M2-APC (0.35 or 1.75 μM) to the reaction resulted in progressively enhanced topo IIα plasmid relaxation activity (lane 3 and 4). M2-APC did not have plasmid relaxation activity in the absence of topo IIα (lane 5). (D) Using a higher concentration of topo IIα (0.7 μM), the addition of BSA (0.7 μM) did not enhance the plasmid relaxation activity, but rather slightly inhibited it (compare lanes 2 and 3). M2-APC (0.7 μM) enhanced the plasmid relaxation activity (lane 5).

inhibited topo IIα activity (Figure 2.6D). These *in vitro* assays provide additional support for a functional interaction between APC and topo IIα. Taken together, our data implicate topo IIα as a mediator of abnormal DNA morphology and G2 arrest associated with exogenous M2-APC expression.

Discussion

In this paper we identify a novel interaction between the tumor suppressor protein APC and topo IIα. This interaction was demonstrated by colocalization, reciprocal co-immunoprecipitation, FRET, and functional assays. Topo IIα interacts with the 15-amino acid repeat region of APC (M2-APC, amino acid 959-1338). When over-expressed in colon cancer cell lines, M2-APC located predominantly to the nucleus. Cells expressing M2-APC displayed abnormal nuclear morphology and were inhibited in the G2 phase of the cell cycle. Although M2-APC could bind β-catenin, we found no evidence supporting a role for β-catenin in the observed phenotypes. Our data suggest a novel role for nuclear APC in the regulation of cell cycle progression, potentially through an interaction between topo IIα and the 15 amino acid repeat region of APC.

The GFP-M2-APC protein was not expected to locate predominantly to the nucleus. Although endogenous full length APC exists in both cytoplasm and nucleus (20, 30), the classic NLSs that are thought to facilitate nuclear localization of APC (28) are not present

in the M2-APC fragment. The M2-APC region is retained in most truncated forms of APC associated with colorectal cancer and these truncated APC proteins are capable of nucleo-cytoplasmic shuttling (31). However, the nuclear import ability of these truncated APC proteins has been attributed to the armadillo repeat region (aa 334-625), not the 15 amino acid repeat region (32). With an estimated molecular weight of ~70 kDa, GFP-M2-APC is too large to enter the nucleus by diffusion which has a size limitation of 30-50 kDa (33-35). Therefore, M2-APC likely contains a nonstandard NLS or is carried into the nucleus by a nuclear binding partner. Because here we show that M2-APC binds to the abundant nuclear protein topo IIα, it is possible that nuclear entry of M2-APC is facilitated in part by topo IIα. APC has also been reported to bind Proliferating Cell Nuclear Antigen (PCNA) through the M2 region (36) and we have confirmed that PCNA co-immunoprecipitates with M2-APC (data not shown). We reason that other nuclear proteins, such as PCNA, might contribute to the nuclear entry of M2-APC as well.

As an abundant nuclear protein, topo IIα is involved in a number of processes throughout the cell cycle, including transcription, DNA replication, chromatin recombination and organization, and regulation of the G2 decatenation checkpoint (9, 11-15). It is possible that the topo IIα we consistently found associated with endogenous APC and with the M2-APC fragment (Figure 2.1B & 2.6B) corresponds to a specific pool of topo IIα involved in regulation of the G2-M transition. Other pools of

topo IIα might be positioned near replicating heterochromatin during S phase (37), tightly associated with DNA to aid chromosome assembly at the G2-M transition (38-40), responsible for transcription regulation at G1/S (41), or involved in chromosome segregation at the exit of mitosis (16, 42, 43). One explanation for the rather modest amount of topo IIα that co-precipitated with endogenous APC is that the interaction between APC and topo IIα is transient, only occurring at a specific point of the cell cycle.

APC is not the first tumor suppressor protein to interact with topo IIα. BRCA1 (44, 45), RB (46), and p53 (47) each bind to topo IIα and thereby effect topo IIα functions. A recent report also links topo IIα to the oncoprotein β-catenin and its transcription co-factor TCF (41). Over-expression of topo IIα led to enhanced β-catenin /TCF transcription activity, while expression of a constitutively active β-catenin led to increased topo IIα activity measured in a whole cell lysate. We predict that β-catenin /TCF associates with a topo IIα pool that is involved in transcription regulation at G1/S. It is possible that this interaction is indirect and mediated by APC. APC has been found in association with the enhancer region of genes regulated by β-catenin/TCF (48). In contrast, we suspect that the APC/topo IIα interaction we described here is direct. Purified recombinant human M2-APC enhanced the plasmid relaxation activity of purified topo IIα in our *in vitro* assays which lacked other potential linker proteins (Figure 2.6, C and D). Moreover, M2-APC does not appear to bind directly to DNA as

determined by gel shift assays (data not shown). We propose that the pool of topo IIα that interacts with APC to impact G2 cell cycle transition is separate from the pool involved in β-catenin mediated transcription.

We report that the topo IIα protein level was higher in cells expressing GFP-M2-APC than in GFP-expressing cells (Figure 2.6A). This observation is consistent with previous reports that topo IIα levels peak in G2 (49). We also report that purified M2-APC enhances topo IIα activity in an *in vitro* relaxation assay (Figure 2.6C and D). Cell cycle progression from G2 to M is likely dependent on the maintenance of topo IIα activity at a precise level. Indeed, Epstein-Barr Virus kinase BGLF4 both stimulates topo IIα activity and induces premature chromosome condensation similar to the abnormal DNA morphology we report with M2-APC expression (50). Furthermore, protein kinase C δ regulates topo IIα activity specifically during S-phase with aberrant activation of topo IIα by protein kinase C δ leading to apoptosis (51). At the simplest level, we propose that endogenous APC regulates topo IIα activity and thereby facilitates G2-M cell cycle progression.

Topo IIα activity is essential for the chromatin decatenation required before mitosis (16). APC locates near centrosomes during early and late stages of mitosis and has also been implicated in maintenance of chromatin structure (5, 41). Therefore, we cannot exclude the possibility that both APC and topo IIα provide multiple functions throughout

the G2/M transition. We propose that a functional interaction between APC and topo IIα results in regulation of topo IIα activity. Without this regulation, cell cycle progression would be affected. Given that truncated APC found in most colorectal cancers includes the M2-APC region, it is possible that topo IIα regulation is maintained in these cancers. In this scenario, retention of the topo IIα regulatory domain in truncated APC might be essential for cell viability. Alternatively, truncated APC proteins associated with cancer might behave more like the M2-APC fragment and be unable to effectively regulate topo IIα activity. The resulting stimulation of DNA cleavage by topo IIα would be predicted to induce chromosome instability, a hallmark of cancer.

Taken together, we propose a novel function for the tumor suppressor APC in regulation of the G2-M cell cycle transition, potentially through interaction with topo IIα. Future investigation will be needed to determine the precise underlying mechanism by which M2-APC promotes G2 cell cycle arrest. Such studies will potentially reveal a novel activity of APC in tumor suppression. This expanded role of the tumor suppressor APC has obvious implications in explaining the deregulation of topo IIα in colon cancer tissue (17) and for the use of topo IIα inhibitors as chemotherapeutic agents to treat colorectal cancer.

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CHAPTER 3

CENTRAL DOMAINS OF ADENOMATOUS POLYPOSIS COLI INHIBIT ENDOGENOUS TOPOISOMERASE IIα ACTIVITY AND ARREST CELLS IN G2

Abstract

Tumor suppressor Adenomatous Polyposis Coli (APC) is a multi-functional protein. Mutations in *APC* are thought to initiate the majority of colorectal cancers. Our previous investigation implicated the 15 amino acid repeat region of APC (M2-APC) in the regulation of the G2-M cell cycle transition through interaction with topoisomerase IIα (topo IIα). We now demonstrate that the 20 amino acid repeat region of APC (M3-APC) also interacts with topo IIα. Expression of M3-APC in cultured colonic cells causes cell accumulation in G2, similar to expression of M2-APC. However, in cells with reduced endogenous topo IIα, expression of either M2- or M3-APC does not result in G2 arrest. Therefore, the ability of M2- and M3-APC to induce G2 arrest appears dependent on normal expression of topo IIα. Expression of M2- or M3-APC in cultured cells leads to reduced activity of endogenous topo IIα. This finding is consistent with previous reports that inhibition of topo IIα activates the G2 decatenation checkpoint, leading to G2 cell cycle arrest. Although both central domains of APC are also involved in down-regulation of β-catenin, this G2 cell cycle arrest appears to be independent of

β-catenin. Together, our data establish that two central APC domains interact with topo IIα, inhibit topo IIα decatenation activity, and cause G2 cell cycle arrest. These findings implicate APC in regulation of the G2 decatenation checkpoint.

Introduction

Adenomatous Polyposis coli (APC) is an established tumor suppressor protein. Mutation of the *APC* gene is considered an initiating event in over 80% of all colorectal cancers (1). The ability of APC to suppress Wnt signaling by targeting β-catenin for ubiquitin-mediated proteasomal degradation is considered critical for APC to suppress tumorigenesis (2-6). Although alteration of either APC or β-catenin is sufficient for initiating intestinal tumors, APC likely suppresses tumor development through pathways besides inhibiting Wnt signaling [see review (7)].

Among the multiple functions of APC that have been identified are spindle formation and microtubule stabilization (8-11), long patch base excision repair (12), and cell cycle regulation (8, 13-18). Mutations in *APC* have also been implicated in chromosomal instability and aneuploidy in early FAP polyps (19-21). The function of APC, as well as the underlying mechanism for many of these processes, is only beginning to be understood.

Recently, we have identified topoisomerase IIα (topo IIα) as a novel APC binding partner that participates in regulation of the G2-M cell cycle transition (22). Topo IIα is a type II topoisomerase that catalyzes DNA topology change throughout the cell cycle (23-26). Topo IIα has also been considered a critical regulator at the G2/M decatenation checkpoint during cell division (27). Inhibition of topo IIα activity leads to activation of the G2 decatenation checkpoint resulting in G2 arrest (27). Topo IIβ with similar activity as topo IIα, is believed to be dispensable in cell cycle control because topo IIβ-null cell lines remain viable (28-30).

Previously, we found endogenous APC interacted with topo IIα but not with topo IIβ. Expression of a central fragment of APC that binds topo IIα led to abnormal nuclear morphology and cell cycle inhibition in G2 in various colon cancer cells lines (22). Although β-catenin is capable of binding to this central fragment of APC, β-catenin appeared not involved in this process. We concluded that nuclear APC interacts with topo IIα and thus might be involved in the regulation of cell cycle progression.

In the current study, we identify a second domain in the central portion of APC that specifically binds to topo IIα but not topo IIβ. Both APC central domains dramatically impact the activity of topo IIα *in vitro* and *in vivo*. Expressing each of the APC domains capable of topo IIα interaction leads to cell cycle arrest in G2. Using the HL-60/MX2 cell line, a variant of human promyelocytic leukemia cell line HL-60 with notably reduced

levels of topo IIα, we demonstrate that APC-mediated cell cycle arrest happens only in cells with normal expression of endogenous topo IIα. Our data suggest APC participates in the topo IIα-dependent regulation of the G2-M transition.

Materials and Methods

Cell culture and DNA constructs

HCT116βw cells (a generous gift from Dr. Bert Vogelstein) were grown in McCoy's 5A medium (Gibco) supplemented with 10% FBS (Hyclone). HL60 cells (ATCC) were grown in Iscove's Modified Dulbecco's Medium (ATCC) supplemented with 20% FBS (Hyclone). HL60/MX2 (ATCC) were grown in RPMI 1640 media (Cellgro) supplemented with 10% FBS. Expression constructs for APC fragments fused to GFP were kindly provided by Dr. Naoki Watanabe and have been described previously (9). His and S dual-tag fused M2-APC was made as described (22). To generate recombinant N-terminal His and S dual-tag fused APC fragment M3, the corresponding cDNA for APC (amino acid 1327-2058) was amplified using PCR and subcloned into a pET-30a(+) vector.

Immunoprecipitation and immunoblots

HCT116βw cells were transfected using Lipofectamine2000 reagent according to the manufacturer's protocol (Invitrogen). Immunoprecipitation (IP) and immunoblots (IB) were performed using anti-GFP pAb (Invitrogen) as described (22). Immunoblots were probed with the following antibodies: anti-β-catenin (1:2000, Sigma), anti-topo IIα (1:1000, Research Diagnostics, Inc.), anti-topo IIβ (1:1000, Santa Cruz), anti-GFP pAb (1:1000, Invitrogen) and anti-α-tubulin (1:2000, Oncogene).

Electroporation and FACS analysis

Cells grown on plastic were treated with trypsin to obtain a single cell suspension. A total of 2μg of GFP, GFP fused M2-, or M3-APC plasmid were electroporated using Nucleofector I (Amaxa) according to the manufacturer's protocol. Electroporation programs used are: HCT116βw (program D-32), HL60 (program T19), and HL60/MX2 (program X-03). 48hrs post transfection, single cells in suspension were stained with 0.5μg/ml Hoechst 33342 (Invitrogen) for 30min at 37℃. FACS analysis was performed using both UV and 488nm lasers on a 5-laser BD LSRII flow cytometry (BD Bioscience). 10,000 GFP-positive cells were collected for each sample. Data were analyzed using BD FACSDiva Software (BD Bioscience) and plotted using WinMDI 2.9.

Recombinant proteins and topo IIα decatenation assays

Recombinant S-tag fused M2- and M3-APC were generated as described (22). BSA (Sigma) was diluted in S-tagged APC protein dilution buffer (20mM Hepes pH 7.8, 100mM NaCl). Recombinant human topo IIα and topo IIβ were made as described (31, 32). *In vitro* topo IIα decatenation assays were performed as described (24). Nuclear extracts were made exactly as previously described (33). Nuclear extracts were frozen and kept in aliquots in liquid nitrogen if not immediately used after preparation. Immunoblots were performed first to normalize topo IIα protein levels in all nuclear extracts. Then ~1μg of nuclear extract with normalized topo IIα protein level was used for decatenation activity assays.

Antibodies and immunofluorescence

Cells transfected with GFP or GFP-fused M3-APC were fixed with 4% Paraformaldehyde, and immunostaining was performed using anti-phospho-histone H3 (1:500, Upstate) as described (34). 100 GFP-positive cells were randomly chosen, and only cells also positive for phospho-histone-H3 were counted. The number of mitotic cells was calculated as an average ± s.d. of three independent experiments.

Results

A central domain of APC capable of β-catenin down-regulation also binds topo IIα

As a critical tumor suppressor in the colon, APC has been widely studied, and multiple APC functions have been identified in different stages of the cell cycle [see review (35)]. Previously, we found full-length endogenous APC specifically interacts with topo IIα and expression of the 15 amino acid repeat region of APC (M2-APC) that interacts with topo IIα results in altered DNA morphology and accumulation of cells in the G2 phase (22). For this prior analysis, technical difficulties in protein expression and purification limited the domains of APC that were characterized in full.

In the present study, we report that both M2-APC and the 20 amino acid repeat region of APC (M3-APC) interact with topo IIα. Topo IIα specifically co-precipitates with both GFP-fused M2- and M3-APC (Figure 3.1, top blot). However, topo IIβ does not co-precipitate with either APC fragment under the same experimental conditions (Figure 3.1, second blot). M2-APC encompasses APC amino acids 959-1338 while M3-APC contains amino acids 1211-2075. These two regions together comprise the entire 15 amino acid and 20 amino acid repeat regions of APC, respectively, which mediate β-catenin binding and downregulation. Not surprisingly, β-catenin co-precipitated with both M2- and M3-APC (Figure 3.1, third blot).

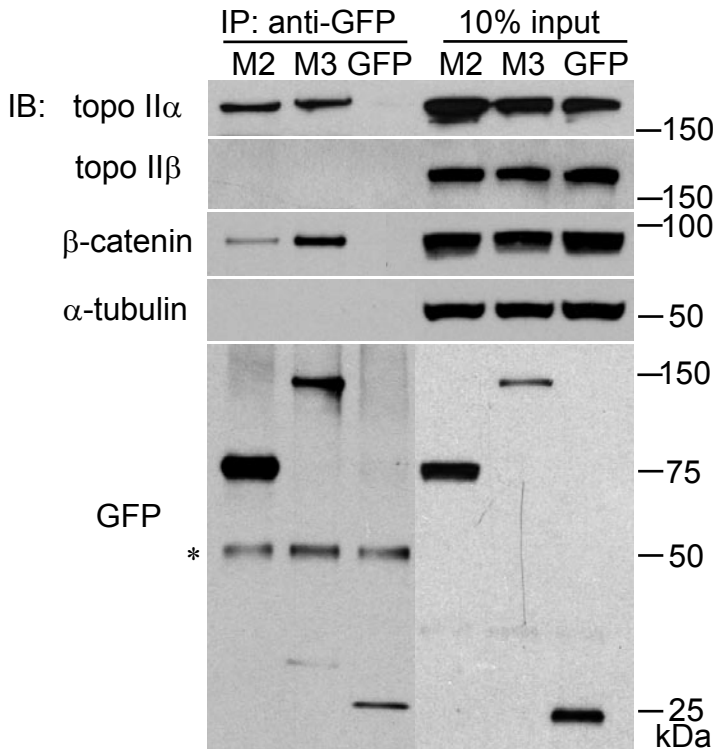

Figure 3.1
Both 15aa and 20aa repeat regions of APC interact with topo IIα. Topo IIα co-immunoprecipitates with both M2-APC and M3-APC, but not with GFP from M2- or M3-APC or GFP expressing HCT116βw cells. 10% input equals 10 μg total protein. Topo IIα does not co-precipitate with M2- or M3-APC, whereas β-catenin does. Blots probed for α-tubulin served as a loading control for the input samples and a negative control for the immunoprecipitations. (*) marks migration of antibody heavy chain. Representative blots from three independent experiments are shown.

Expression of M2- or M3-APC results in G2 cell cycle arrest

Like M2-APC (22), M3-APC binds topo IIα in cells (Figure 3.1). Thus, we expressed M3-APC in HCT116βw cells to examine if M3-APC also caused a delay in the cell cycle. As predicted, cells expressing M3-APC as well as M2-APC progressively accumulate in the G2/M phases of the cell cycle, compared to cells expressing GFP only (Figure 3.2 A and B, Table 3.1). By 72 hours, cells expressing M3-APC displayed a 2-fold increase in the G2/M population and a 31% reduction in the S phase population, while cells expressing M2-APC displayed a 2.4-fold increase in the G2/M population and a 77% reduction in the S phase population, compared to GFP expressing cells. Overall, expression of M2- or M3-APC consistently led to cell cycle arrest in G2/M. Of note, the reduction in S phase cells seen upon expression of M2-APC and to a lesser extent M3-APC, suggested a second cell cycle delay prior to S phase, likely in G1. This apparent delay in G1 is consistent with a previous observation that APC controls the G1-S transition (15). M2-APC expression elicits G2 phase cell cycle arrest rather than arrest in M (22). The percentage of M3-APC expressing cells undergoing mitosis (4%), as indicated by phospho-histone H3 positive staining, is similar ($p>0.05$) to that in GFP-expressing cells (3%) (Figure 3.3 A and B). Therefore, as previously reported for M2-APC, the expanded G2/M population of M3-APC expressing cells determined by

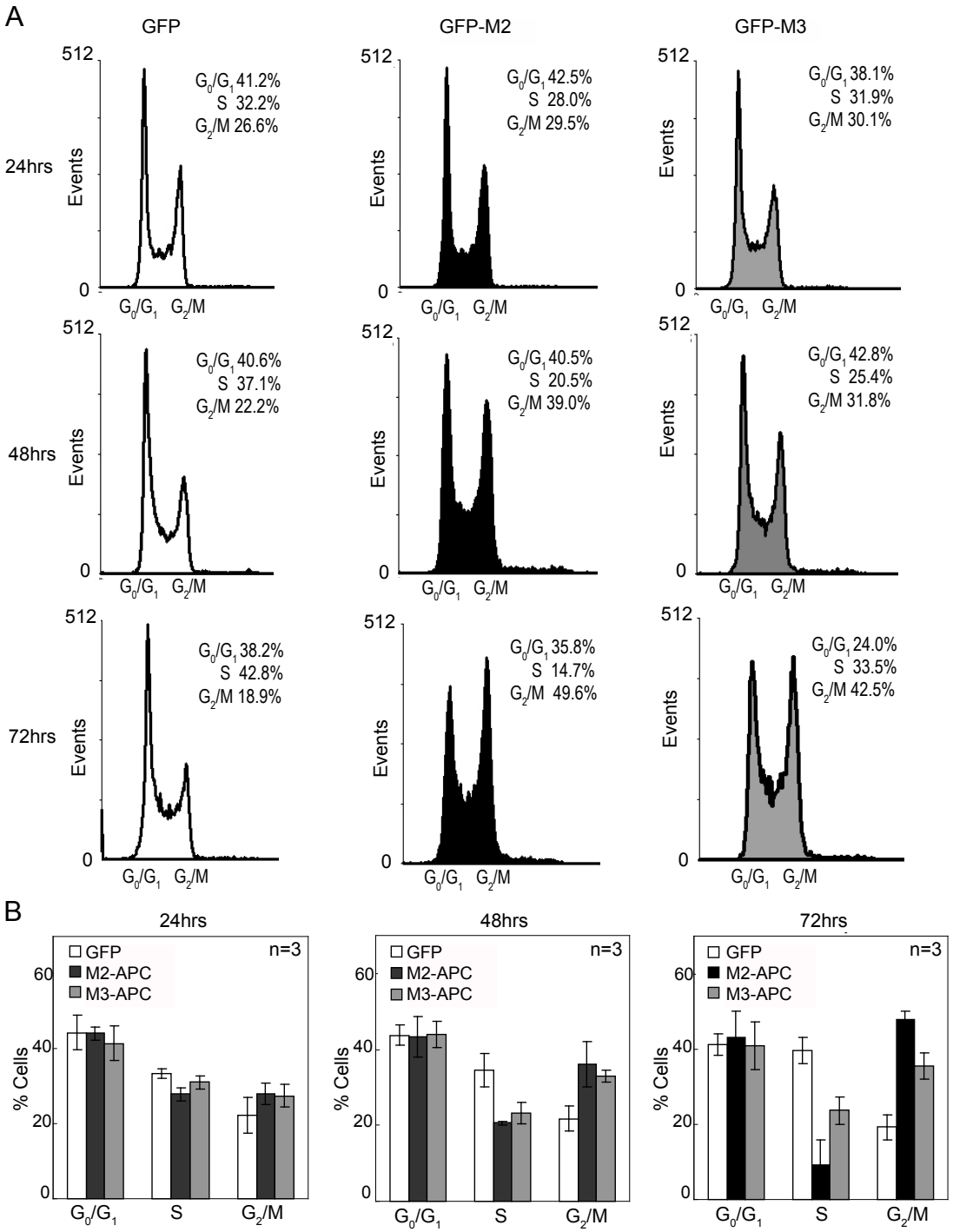

Figure 3.2

Cells expressing M2- or M3-APC progressively accumulate in G2/M. (A) Histograms showing representative FACS displays of cell cycle distribution assessed by Hoechst blue staining at 24, 48, and 72hrs post-transfection with expression constructs for GFP fused M2-, M3-APC, or GFP. Only GFP positive cells are displayed. (B) Bar graphs show FACS based cell cycle distribution at 3 timepoints post transfection from three independent experiments. By 72hrs post-transfection, the fraction of M2-APC and M3-APC expressing cells in G2/M increased by 2.4-fold and 2-fold, respectively; and the S phase decreased by 77% and 31% , respectively.

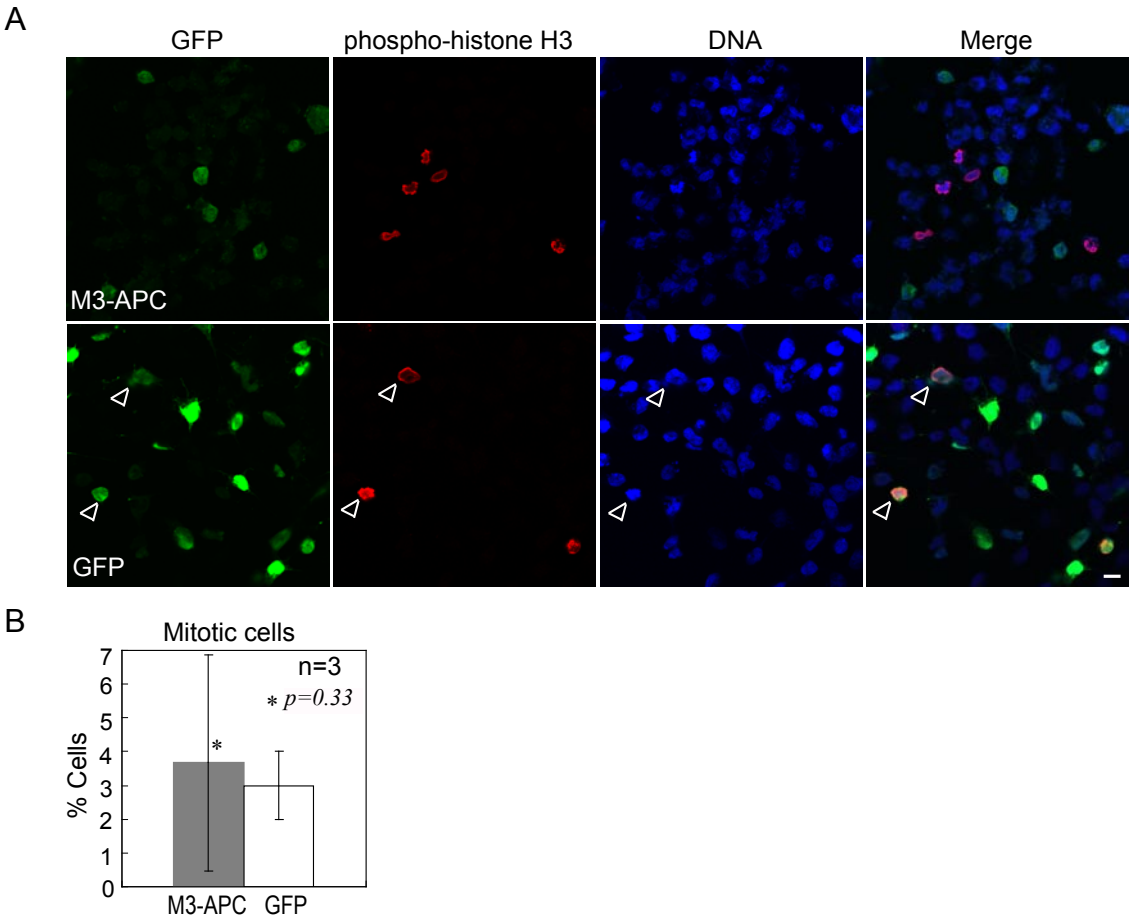

Figure 3.3
Cells expressing M3-APC exhibit normal M phase cell cycle distribution. (A) Cells expressing GFP or GFP-M3-APC were analyzed for expression of mitotic marker phospho-histone H3. A small portion of GFP-expressing cells shown were double positive for phospho-histone H3. (B) Scoring 100 GFP-positive cells from each of three independent experiments revealed no change in the mitotic population when cells expressed M3-APC. Bar, 10 μm.

Table 3.1 Cell cycle distribution in cells expressing GFP, M2-APC, or M3-APC

	24 h	G0/G1 (%)	S (%)	G2/M (%)	Aneuploid
24hrs	GFP	44.3 +/- 4.7	33.4 +/- 1.3	22.3 +/- 4.8	1.7 +/- 1.21
	M2-GFP	44.1 +/- 1.7	27.9 +/- 1.6	28.0 +/- 2.8	2.8 +/- 0.5
	M3-GFP	41.5 +/- 4.6	31.0 +/- 1.6	27.5 +/- 3.1	2.6 +/- 0.6
48hrs	GFP	43.8 +/- 2.8	34.5 +/- 4.3	21.7 +/- 3.3	1.5 +/- 0.8
	M2-GFP	43.3 +/- 5.4	20.6 +/- 0.6	36.1 +/- 5.9	5.5 +/- 1.1
	M3-GFP	43.9 +/- 3.5	23.2 +/- 2.9	32.9 +/- 1.6	4.1 +/- 0.7
72hrs	GFP	41.1 +/- 2.8	39.7 +/- 3.4	19.2 +/- 3.4	1.7 +/- 0.8
	M2-GFP	43.1 +/- 6.9	9.2 +/- 6.6	47.7 +/- 2.5	**9.0** +/- 4.9
	M3-GFP	33.6 +/- 9.5	27.2 +/- 6.6	39.2 +/- 3.0	**5.6** +/- 3.0

Transfected cells were stained with Hoechst blue, and the cell cycle distribution G0/G1 (2N), S (between 2N and 4N), and G2/M (4N) was determined by FACS at three time points post transfection. Aneuploid cells are shown as a percentage of the total cycling cell populations. For each transfection, 10,000 GFP-positive cells were analyzed by FACScan. Table shows the average from three independent experiments.

FACS analysis represents an arrest in G2. Together, these results demonstrate that M3-APC and M2-APC are each capable of inducing G2 cell cycle arrest when expressed in colon epithelial cells.

Cells with reduced topo IIα levels do not arrest in G2 following expression of either M2- or M3-APC

Based on our data, we propose a model whereby exogenous M2- or M3-APC interacts with endogenous topo IIα resulting in G2 cell cycle arrest. Ideally, in order to show this APC-mediated cell cycle perturbation is dependent upon topo IIα, we should eliminate topo IIα from the analyzed cells. Unfortunately, topo IIα is an essential protein throughout the cell cycle, and perturbation of its activity typically results in inhibition of normal cell proliferation. No cultured mammalian cell line completely lacking topo IIα has been reported in the literature. In a cell line in which topo IIα can be conditionally deleted, complete removal of topo IIα leads to acute cell cycle delay in G2 followed by cell death (36). However, there are cell line variants with greatly reduced topo IIα levels which are comparatively resistant to topo II targeting drugs. HL-60/MX2 cells, a variant of the human promyelocytic leukemia cell line HL-60, were selected for resistance to the topo II inhibitor mitoxantrone (37). HL-60/MX2 cells express truncated topo IIα with reduced activity and at a reduced level compared to the parental HL-60 cells (38-40)

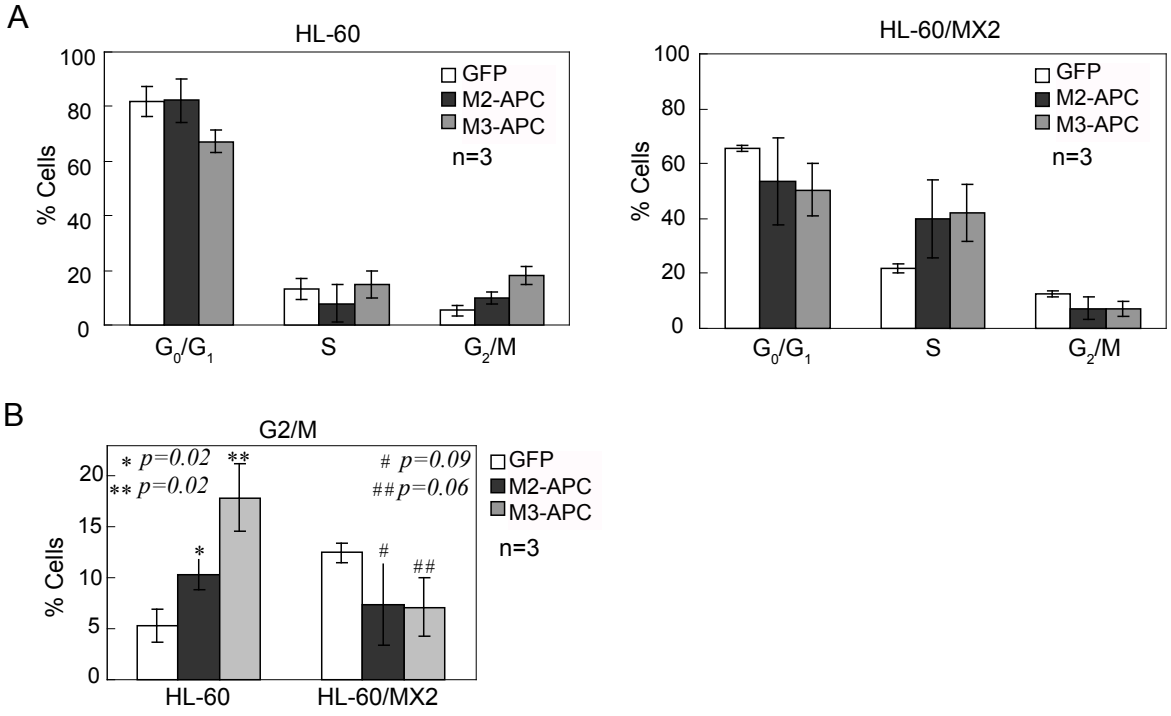

Figure 3.4
Expression of M2- or M3-APC causes G2/M accumulation in HL60 but not HL60/MX2 cells with reduced topo IIα. (A) Cell cycle distribution of HL-60 and HL-60/MX2 cells expressing M2- or M3-APC. (B) Detailed bar graph showing only G2/M population from both cells lines in (A). Parental HL60 cells exhibit a 1.9 fold increase in G2/M when expressing M2-APC (*,*p*= 0.02) and a 3.4 fold increase when expressing M3-APC (**, *p*=0.02), compared to cells expressing only GFP. HL60/MX2 cells do not accumulate in G2/M when expressing M2- (#, *p*=0.09) or M3-APC (##, *p*=0.06), compared to cells expressing only GFP. For both cell lines, 10,000 GFP-positive cells were analyzed from three independent experiments at 48hrs post transfection.

Table 3.2 Cell cycle distribution of parental HL60 and HL60/MX2 cells expressing GFP, M2-APC, or M3-APC

HL60	G0/G1 (%)	S (%)	G2/M (%)
GFP	81.8 +/- 5.4	13.0 +/- 3.8	5.2 +/- 2.0
M2-GFP	82.2 +/- 7.8	7.8 +/- 6.8	10 +/- 2.1
M3-GFP	67.3 +/- 4.1	14.8 +/- 5.1	17.9 +/- 3.3

HL60/MX2	G0/G1 (%)	S (%)	G2/M (%)
GFP	65.7 +/- 1.0	21.7 +/- 1.8	12.6 +/- 1.0
M2-GFP	53.3 +/- 15.9	39.4 +/- 14.2	7.3 +/- 4.0
M3-GFP	50.5 +/- 9.7	42.3 +/- 10.1	7.2 +/- 2.8

Cell cycle distributions were determined by FACS analysis of Hoechst blue stained GFP, M2- and M3-APC expressing cells at 48 hrs post transfection. For each transfection, 10,000 GFP-positive cells were analyzed by FACS. Table shows the average from three independent experiments.

and almost no topo IIβ. When M2- or M3-APC was expressed in parental HL-60 cells, the G2/M population increased significantly (Figure 3.4 A left graph and B, Table 3.2). M2-APC expression resulted in nearly a doubling of the G2/M population, while M3-APC expression resulted in tripling of the G2/M population. The G2 arrest in HL-60 cells indicates that the impact of M2- and M3-APC on the cell cycle is not restricted to colon epithelial cell lines. HL-60 cells grow in suspension and divide less frequently than the colon epithelial cells. Thus, it was not surprising to find the majority of cells in the G0/G1 phases of the cell cycle. In contrast, HL-60/MX2 cells expressing M2- or M3-APC showed no increase in the G2/M population (Fig. 3.4 A right graph and B, Table 3.2). Rather, the slight decrease in G2/M and increase in the S population of M2- and M3-APC expressing cells was not significantly different from the GFP-expressing HL-60/MX2 cells ($p>0.05$). Taken together with our observation that M2- and M3-APC interact preferentially with topo IIα rather than topo IIβ (Figure 3.1), we propose that cell cycle arrest in cells expressing M2- and M3-APC is dependent on normal expression of topo IIα.

Expression of M2- or M3-APC inhibits topo IIα activity in cells

Inactivation of topo IIα leads to G2 cell cycle arrest, most likely by activating the G2 checkpoint (27). To examine if the G2 cell cycle delay caused by M2- or M3-APC expression resembled activation of the G2 checkpoint, we extracted M2- or M3-APC

expressing HCT116βw cell nuclear lysates containing topo IIα to assess the activity of endogenous topo IIα. Topo IIα protein levels were used to standardize the amount of each nuclear extract tested in DNA decatenation assays (Fig. 3.5 B, top blot). Nuclear lysates from HCT116βw cells expressing GFP had similar topo II decatenation activity as lysates from non-transfected HCT116βw cells (Fig. 3.5 A, lanes 3 and 4). Nuclear extracts from cells expressing M2- and M3-APC exhibited reduced topo II decatenation activities compared to extracts from untransfected or GFP-expressing cells (Fig. 3.5 A, lanes 5 and 6). There was only minimal topo IIβ protein in the nuclear extracts, and this level did not correlate with the DNA decatenation activity of the samples (Fig. 3.5 B, middle blot). M2- and M3-APC were present in these nuclear lysates (Fig. 3.5 B, bottom blot). We conclude that this nuclear M2- or M3-APC is responsible for inhibition of endogenous topo IIα activity.

Discussion

In this study we identify a novel topo IIα binding domain (M3) in the central part of APC. Cells expressing M2- or M3-APC were inhibited in the G2 phase of the cell cycle. Endogenous topo IIα activity in both M2- or M3-APC expressing cells was greatly reduced. Although M3-APC could bind β-catenin, we found no evidence supporting a role for β-catenin in the observed phenotypes. Our data indicate the central region of APC interacts with and inhibits topo IIα, thus regulating G2-M cell cycle progression.

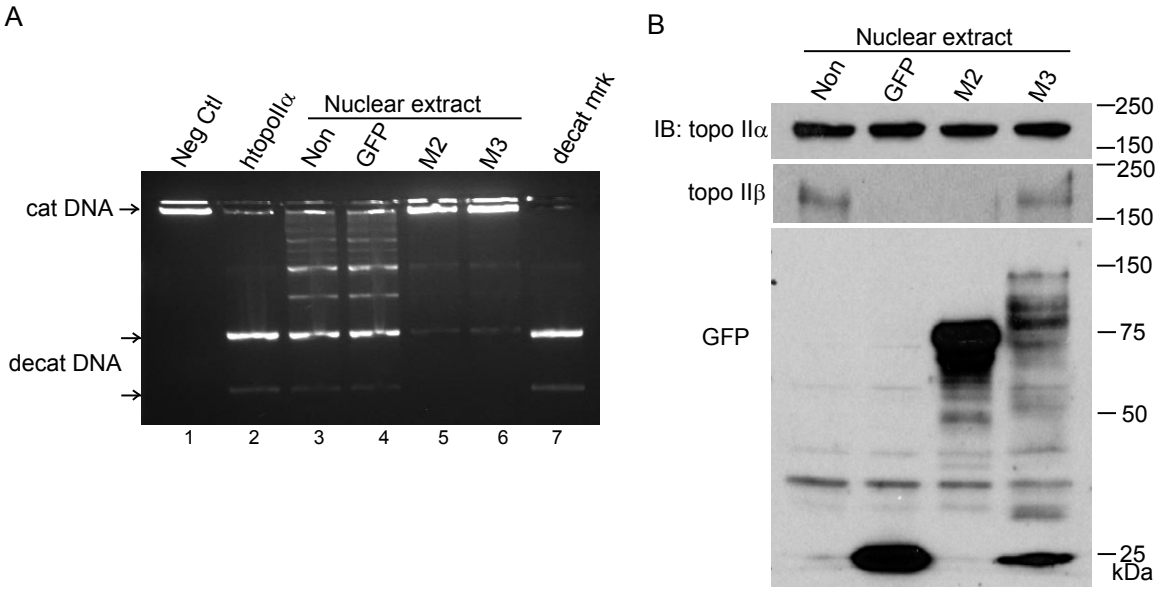

Figure 3.5
Exogenous expression of M2- or M3-APC inhibits topo II decatenation activity in nuclear extracts. (A) Nuclear extracts from HCT116βw cells (lane 3) or cells expressing GFP (lane 4) possess decatenation activity. Recombinant purified human topo IIα provides a positive control (lane 2). Cells expressing M2- or M3-APC show reduced decatenation activity (lanes 4 and 5). One μg of total nuclear extract was used for each assay. Representative assays from three independent experiments are shown. cat DNA, catenated kDNA; decat DNA, decatenated kDNA. (B) Immunoblots reveal comparable topo IIα protein in each nuclear extract used in (A) (top panel). Minimal topo IIβ was detected on replicate blots (middle panel). GFP and GFP-fused M2-APC were abundant in the nuclear extracts (bottom panel). Full length GFP-M3-APC (~150kDa) was also present, along with multiple degradation products. Six μg of total nuclear extract were loaded in each lane. Representative blots from three independent experiments are shown.

HCT116βw cells express full length endogenous APC as well as wild-type β-catenin. Since HCT116βw cells have been altered from the parental HCT116 line such that they no longer express the mutant β-catenin allele (encoding a stabilized version of β-catenin) and they do not have high endogenous β-catenin activity (41). That there is only a slight reduction in β-catenin activity in HCT116βw cells expressing M3-APC (data not shown) is likely because endogenous APC is already eliminating most of the β-catenin available for destruction. M2- and M3-APC both inhibit topo IIα activity and arrest cells in the G2 phase, indicating their functional similarity in cell cycle modulation. The phenotype of G2 arrest caused by expression of M2-APC is independent of β-catenin (22). Therefore, it is likely that the nearly identical phenotype of G2 arrest caused by M3-APC expression also does not require β-catenin.

Previously, we reported that purified recombinant M2-APC (amino acids 999-1326) stimulated the activity of purified topo IIα in plasmid relaxation and decatenation assays (22). Purified recombinant M3-APC (amino acids 1327-2058), which does not overlap with the purified M2-APC fragment, also enhanced the topo IIα decatenation activity *in vitro* (Figure 3.6 A). In contrast, we showed that expression of M2- or M3-APC in cells inhibits endogenous topo IIα activity in nuclear lysates (Figure 3.5 A). It is not obvious how to reconcile these two findings. Given the abundance of topo IIα in the soluble nuclear lysates (Figure 3.5 B), it appears that over-expressed M2- or M3-APC does not

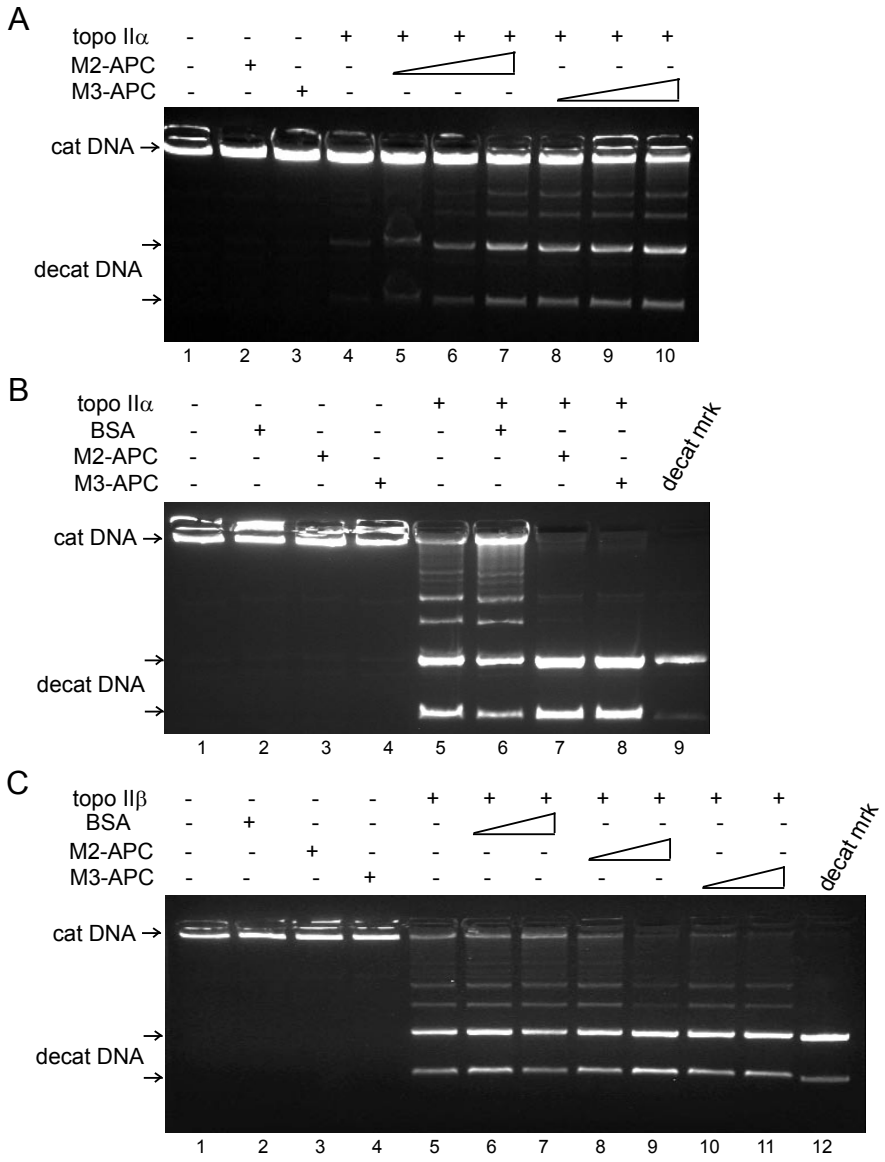

Figure 3.6

Recombinant M2- and M3-APC enhance topo IIα activity *in vitro*. (A) Purified recombinant human topo IIα was used at a concentration (0.12 μM) which could slightly decatenate catDNA (lane 4). Addition of increasing amounts (0.12, 0.24, and 0.6 μM) of purified recombinant M2-APC (lanes 5-7) or M3-APC (lanes 8-10) resulted in progressively enhanced topo IIα activity. M2-, or M3-APC (0.6 μM) alone did not display any decatenation activity. (B) Using a higher concentration of topo IIα (0.18 μM), the addition of M2- or M3-APC (0.18 μM) also enhanced topo IIα activity. In contrast, negative control BSA (0.18 μM) did not enhance the activity of topo IIα, but rather slightly inhibited it (lanes 6-8). (C) Purified recombinant human topo IIβ (0.018 μM) decatenates catDNA (lane 5). Addition of M2- or M3-APC (0.018 and 0.09 μM) minimally enhanced topo IIα activity (lanes 8-11). Addition of negative control BSA (0.018 and 0.09 μM) results in no difference in topo IIα activity (lanes 6 and 7). BSA, M2-, or M3-APC (0.09 μM) did not display any decatenation activity in the absence of topo IIα (lanes 2-4). (A-C) Representative assays from three independent experiments are shown. cat DNA, catenated kDNA; decat DNA, decatenated kDNA.

reduce topo IIα activity simply by causing precipitation of topo IIα, thus rendering it insoluble. One possibility is related to conditions that promote DNA catenation verses decatenation. Proteins such as histones, that promote DNA condensation, shift the equilibrium from decatenation to catenation (42, 43).Since nuclear extracts may contain histones and histone-like proteins, the reduction of decatenated DNA seen in the topo IIα activity assay using nuclear extracts from cells expressing M2- and M3-APC might represent a re-catenation of the decatenated kinetoplast DNA (Figure 3.5A, lanes 5 and 6). However, substituting plasmid DNA for the kinetoplast DNA in the same assay did not result in DNA catenation, but rather, regular laddering of relaxed plasmid DNA (data not shown). Therefore, M2- or M3-APC expression does not appear to promote DNA condensation as a means to induce DNA catenation.

After excluding the situations stated above, we were left with three possibilities. First, the differences observed using purified recombinant proteins versus the mammalian nuclear extracts might stem from the lack of post-translational modification of the purified components expressed in a non-mammalian (note: our topoisomerase IIα is expressed in yeast, not bacteria) system. Second, topo IIα modifications including SUMOylation, ubiquitination and phosphorylation are seen in cells (42-45). Although the exact functions of these modifications are not clear, it is possible that expression of M2- or M3-APC in cells modulates some of these post-translation modifications of topo IIα

and thus alters cellular topo IIα activity. Third, the complete composition of the APC-topo IIα complex has not been determined. Other APC-interacting proteins in this complex could potentially serve to inhibit topo IIα activity. Future analysis of full-length APC and its impact on topo IIα activity both *in vivo* and *in vitro* will help address this discrepancy. We predict that interaction of full length APC with topo IIα will result in inhibition of topo IIα activity in cells.

Here we show that M2- and M3-APC interact with topo IIα but not topo IIβ in cells (Figure 3.1). This finding is consistent with our previous observation that full-length endogenous APC only interacts with topo IIα but not topo IIβ (22). Purified M2- and M3-APC each slightly elevated topo IIβ activity (Figure 3.6 C, lanes 8-11), an effect much less than with topo IIα. In addition, it is possible that in a reconstituted system using high concentrations of purified components, M2- and M3-APC can interact with topo IIβ and thereby affect its activity. However, M2- and M3-APC do not complex with topo IIβ (Figure 3.1). Successful generations of cell lines and mouse models completely lacking topo IIβ (28-30) suggest that topo IIβ is dispensable for cell cycle progression. Thus, it appears that APC-mediated G2 cell cycle arrest is independent of topo IIβ.

Topo IIα protein levels fluctuate during different stages of the cell cycle, peaking in G2 (46). However, the specific activity of topo IIα remains constant throughout the cell cycle (47-49). Thus, it is likely that the dramatic reduction in topo IIα activity in M2- or

M3-APC expressing cells did not result from changes in the specific activity of topo IIα. Rather, we propose that expression of M2- or M3-APC leads to inhibition of topo IIα activity, which causes G2 arrest. A variety of topo II inhibitors have been shown to delay cells in G2 by activating the G2 decatenation checkpoint (27). It is possible that the M2- and M3-APC fragments, and even full-length APC, serve to activate the G2 decatenation checkpoint by inhibiting topo IIα. The G2 decatenation checkpoint is vital for cell cycle control and genomic integrity. Cells lacking the G2 decatenation checkpoint become aneuploid (50, 51). In our study, the population of aneuploid cells expressing M2- or M3-APC steadily increased from 24 to 72 hrs post-transfection (Table 3.1). This accumulation of aneuploid cells possibly results from inhibiting topo IIα activity in the small percentage of mitotic cells that escape the G2 decatenation checkpoint.

As a critical component of the G2 decatenation checkpoint, topo IIα is important for regulation of normal cell proliferation (25, 52). If full length APC acts in a similar manner as the M2- and M3-APC fragments, it would be expected to inhibit topo IIα activity under physiological conditions to suppress tumor growth. In cancer, mutant APC would fail to regulate this process and allow cells to escape the G2 checkpoint, accumulate chromosomal instability, and potentially become aneuploid. Over 60% of FAP polyps displayed aneuploidy (19-21), and mutations in *APC* have been shown to promote aneuploidy in mouse models as well (53, 54). Our results provide a potential

explanation for the presence of aneuploidy in early FAP and *APC*-driven mouse intestinal adenomas. Further studies are required to elucidate the mechanism of APC in topo IIα-dependent G2 checkpoint. These studies will increase our understanding of the molecular factors involved in the pathogenesis of colorectal cancer so that improved diagnostic and treatment strategies can be developed.

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CHAPTER 4

NOVEL ASSOCIATION OF APC WITH INTERMEDIATE FILAMENTS IDENTIFIED USING A NEW VERSATILE APC ANTIBODY

Abstract

Recognized as a key player in suppression of colon tumorigenesis, Adenomatous Polyposis Coli (APC) protein has been widely studied to determine its normal cellular functions. Direct interactions of APC with microtubule component tubulin and microtubule-binding protein EB1 point to a role for APC in spindle formation and migration. Recent studies have shown that APC regulates cell polarity and migration through interactions with the actin cytoskeleton. Therefore, it is of interest to determine if APC also interacts with the third cytoskeletal element: intermediate filaments. In recent years, however, inconsistencies of commercially available APC antibodies have limited the exploration of APC's biological functions. Using a novel APC antibody raised against the 15 amino acid repeat region of APC (APC-M2), we immunoprecipitated endogenous wild-type APC and its binding proteins from HCT116βw cells, which contain wild-type APC and β-catenin. By Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS), we identified 42 proteins in complex with APC. Among the most abundant proteins found to interact with APC were β-catenin and intermediate filament (IF)

proteins lamin B1 and keratin 81. Association of lamin B1 with APC in both cultured cells and human colonic tissue was confirmed by co-immunoprecipitation and colocalization. APC also colocalized with keratins and remained associated with IF proteins throughout a sequential extraction procedure. We also verified the reliability of the new versatile APC antibody in several applications including immunoprecipitation and immunofluorescence. Thus, we introduce a new APC antibody that is useful for immunostaining, immunoblotting and immunoprecipitation. Using this antibody, we present evidence for an interaction between APC and IFs, independent of actin microfilaments or microtubules. Our results indicate that APC associates with all three major components of the cytoskeleton, thus expanding APC roles in the regulation of cytoskeletal integrity.

Introduction

Mutation of the tumor suppressor Adenomatous Polyposis Coli *(APC*) is an early event in colorectal carcinogenesis. Numerous studies on the subcellular localizations and binding partners of APC have indicated a role for APC in a wide variety of cellular functions in normal cells. The most well characterized function of APC is to inhibit Wnt signaling by forming a multi-protein complex which targets cytoplasmic β-catenin for destruction (1-3). More recently, APC has been implicated in the regulation of cytoskeletal integrity.

APC linkage with the actin network has been demonstrated by both direct interaction of APC with actin (4) and also actin-dependent membrane-localization of APC (5). Ectopic expression of APC (6), APC truncating mutations (7), and APC loss (6, 8) all resulted in aberrant cell migration. Identification of APC in a complex with IQGAP (9), a scaffolding protein that binds to growing microtubules and regulates actin filament elongation (10)，provided evidence for a role of APC in cell migration. Depletion of either APC or IQGAP1 inhibited actin polymerization and cell polarization (9). APC also positively affects a Rac-specific GEF, ASEF (11), and thereby activates Cdc42 (12). A truncated APC protein commonly found in colon cancer failed to activate ASEF, potentially contributing to tumor formation (12).

APC interactions with the microtubule cytoskeleton have also been determined. Early localization studies identified APC at the plus ends of microtubules, thus implicating APC in cell migration (13, 14). The functional interaction of APC with the microtubule network is strengthened by the finding that APC directly interacts with tubulin (15, 16) and the microtubule-binding protein, EB1 (17). Depletion of APC was shown to destabilize microtubules and inhibit spindle formation and cellular protrusions, thus compromising cell migration (18).

Actin-containing microfilaments, microtubules, and intermediate filaments (IFs), constitute the three main cytoskeletal elements that coordinate to regulate cell structure,

polarity and migration. IFs function as a scaffold to maintain cell and tissue integrity and defects in IF impact a number of diseases [see review (19)]. In the present study, we provide further evidence for the involvement of APC in the regulation of cytoskeletal structure through interaction with IFs. Using APC-specific polyclonal sera raised against the 15 amino acid repeat region of APC (referred to APC-M2 pAb), we identified interactions of APC with IF proteins lamin B1, lamin B2, keratin 81 and keratin 82. We verified lamin B1 interaction with APC by co-immunoprecipitation as well as by immunofluorescence microscopy in both cultured cells and human colonic tissue. Nuclear lamins are type V IFs that form a spherical mesh lining the nuclear envelope, providing attachment sites for chromosomes and nuclear pores (20). Keratins are type I and II IFs that are predominantly found in epithelial cells, giving cells structural integrity so that they can withstand mechanical stress (21). Identifying an interaction between APC and IFs expands our understanding of the role of APC in cytoskeletal regulation.

Although APC expression and functions have been widely studied in basic research and in clinical settings, recent reports have raised questions about the accuracy and reliability of many commercially available antibodies (22, 23). Therefore, an APC antibody that accurately depicts levels and subcellular localizations of APC in cells and especially in tissues is extremely important. Our characterization of the APC-M2 pAb

validates its use in a variety of applications including detection of APC protein in mouse and human tissues.

Materials and Methods

Cell culture and tissue preparation

HCT116βw cells (a generous gift from Bert Vogelstein) were grown in McCoy's 5A media (Gibco) supplemented with 10% Fetal bovine serum (FBS) (Hyclone). SW480 cells (ATCC) were grown in RPMI 1640 media (Cellgro) supplemented with 10% FBS. HCA46 cells (a generous gift from Ian Tomlinson) were grown in high glucose DMEM media (Gibco) supplemented with 10% FBS. 184A1 cells (a generous gift from Martha Stampfer) are an immortalized human mammary epithelial cell line (24), and were grown in MCDB 170 media (Clonetics) supplemented as described (25). Colon and ileum tissues were removed from an 8 week old male C57/BL6 mouse (Charles Rivers Laboratory) immediately after CO_2 asphyxiation, rolled into "swiss rolls", embedded in OCT medium (Sakura Finetek) and frozen in ethanol-dry ice. Surgical removed normal human colonic tissue was immediately embedded in OCT medium and frozen in ethanol-dry ice.

Antibodies, immunostaining and confocal microscopy

Tissue cryosections or cultured cells were fixed in 4% paraformaldehyde in PBS. Immunostaining of fixed tissue cryosections and cultured cells were performed as described (26, 27). Antibodies used for immunostaining include APC-M2-pAb (1;1000), anti-APC (1:50, ab-7, Calbiochem), anti-β-catenin (1:200, Transduction Lab), anti-lamin B1 (1:50, Oncogene), anti-pan-keratin (1:50, Sigma), Rhodamine-phalloidin (1:500, Invitrogen), and mixture of mAbs of anti-α & β-tubulin (a generous gift from Dave Gard); secondary antibodies of goat anti-mouse IgG FITC (1:200, Molecular Probe), goat anti-mouse IgG Alexa 488 (1:1000, Molecular Probes), goat anti-rabbit IgG Alexa 568 (1:500, Molecular Probes), and goat anti-mouse IgG Alexa 568 (1:500, Molecular Probes). DNA was labeled with Topro-3 (1:1000, Molecular Probes) or DAPI (1:1000, Molecular Probes). Negative controls included incubation with secondary antibody alone, or, for double labeling experiments, secondary antibody with primary antibody of opposing species (eg. Alexa488 anti-mouse IgG with APC-M2-pAb). These negative controls resulted in only minimal signal.

Immunostained cells or tissues were visualized using a PlanNeofluor 40X/1.3 oil objective on a Carl Zeiss Laser Scanning Miscroscope (LSM) 510. Images were analyzed using Zeiss LSM Image Browser Version 4.2. Images of human colon sections were

captured as 2(X) by 3(Y) sub-images and automatically reassembled as one montage image.

Immunoprecipitation and immunoblots

Purified recombinant His-S-M2-APC was resolved on a 4-12% NUPAGE gel (Invitrogen) and stained using a Colloidal Blue Staining Kit (Invitrogen) according to manufacturer's protocol. Immunoprecipitation (IP) and immunoblots (IB) were performed using modified standard protocols. Cells at 90% confluency were lysed in buffer containing 50 mM Tris (pH 7.5), 0.1% NP-40, 100 mM NaCl, 1 mM $MgCl_2$, 5 mM EDTA, protease inhibitor cocktail (Sigma) and Halt phosphatase inhibitor cocktail (PIERCE) on ice for 30 minutes. Cell lysates were sonicated for 10 pulses at level 1 with 10% output, 3 times using an ultrasonic homogenizer (Branson Sonifier 450). Soluble lysates were obtained by centrifugation at 17,000 x g for 20 min at 4^oC. Specific antibodies were first pre-incubated with Protein-A dynabeads (Invitrogen) for 2 hours at room temperature. Then antibody saturated dynabeads were washed with 0.2 M Triethanol amine (pH 8.0) twice and crosslinked using 4 mM DMP (PIERCE) in 0.2 M Triethanol amine (pH 8.0) for 15min. Crosslinking was stopped by adding 1/10 volume of 1M Tris (pH 8.0) and incubating for 2hrs. For each reaction, 1ug of antibody crosslinked to dynabeads was added to 1mg of soluble lysate and incubated overnight at 4°C. Immunoprecipitated proteins were subject to two washes of 15 minutes using lysis

buffer and two washes using PBS-T at 4°C. Immunoblots were probed with: purified anti-APC-M2 (1:5000), anti-lamin B1 (Oncogene, 1:1000), anti-β-catenin (Sigma, 1:2000), and anti-α-tubulin (Oncogene, 1:2000).

Proteomic analysis

Proteins co-precipitated with purified APC-M2 antibody or preimmunue sera were resolved on a 4-12% NUPAGE gel (Invitrogen) and stained using a Colloidal Blue Staining Kit (Invitrogen) according to manufacturer's protocol. The nine gel regions from each sample indicated in Figure 4.3 were excised and combined into experimental and negative control samples. These were then equilibrated in 50 mM NH_4HCO_3, reduced with 3 mM DTT in 100 mM NH_4HCO_3 at 37°C for 15 min, and alkylated with 6 mM iodoacetamide in 50 mM NH_4HCO_3 for 15 min. The gel pieces were then dehydrated with acetonitrile and rehydrated with 15μl of 12.5 mM NH_4HCO_3 containing 0.01μg/μl Modified Trypsin Gold (Promega). Trypsin digestion was carried out for >2 h at 37°C. Peptides were extracted with 60% acetonitrile, 0.1% formic acid, dried by vacuum centrifugation and reconstituted in 30μl of 0.1% formic acid. 5μl of each peptide hydrosylate were analyzed by C18 reverse-phase LC-MS/MS using a Thermo LTQ Ion Trap Mass Spectrometer equipped with a Thermo MicroAS Autosampler and Thermo Surveyor HPLC Pump, nanospray source, and Xcalibur 1.4 instrument control essentially as described (28). Tandem MS data were collected from independent duplicate

experiments, and analyzed with the Sequest algorithm to search the UniRef100 database (Jan 23 2007, 223514 entries) using Xcorr cutoffs of $\geqq 2$ for +2 charged ions and $\geqq 2.5$ for +3 charged ions (29). In addition, the database contained a concatenated decoy database with reversed protein sequences to estimate false discovery rates, which were 5% or below. Proteins identified from negative control were subtracted from experimental sample. Only proteins that appeared in both duplicate experiments were displayed in Table 1 and Supplemental Table 1.

Sequential extraction of cytoskeletal elements

The protocol for sequential extraction of cells was adapted from protocols previously described (30). Briefly, cells were sequentially incubated with cytoskeletal buffer [10mM PIPES pH 6.8, 300mM sucrose, 100mM NaCl, 3mM $MgCl_2$, 1mM EGTA and 0.5% Triton X-100] and extraction buffer [10 mM PIPES, 250 mM ammonium sulfate, 300 mM sucrose, 3 mM $MgCl_2$, and 1 mM EGTA] on ice for 5 minutes, digestion buffer [210 mM PIPES pH 6.8, 300 mM sucrose, 50mM NaCl, 3mM $MgCl_2$, 1mM EGTA, 2 μl /ml DNase and 125 μl/ ml RNase I] at 20^o C for 20 minutes, and 2M NaCl in PBS on ice for 5 minutes. Finally, cells were fixed with 4% paraformaldehyde in PBS and subjected to immunostaining.

DNA constructs, recombinant proteins and fusion protein purification

To generate recombinant N-terminal His and S dual-tag fused APC fragment M2, the corresponding cDNA for APC (amino acid 1000-1326) was amplified using PCR and subcloned into a pET-30a(+) vector. The DNA construct encoding GST-fused M2-APC (amino acid 959-1338) was a generous gift from Kozo Kaibuchi. Both His-S-M2-APC and GST-M2-APC fusion proteins were expressed and purified as described (31, 32).

Affinity-purification of anti-APC-M2 antibody

Anti-APC-M2 rabbit serum was generated by injecting purified S-tag fused M2-APC as the antigen. Serum was acquired and applied to a column of GST-fused M2-APC covalently linked to NHS-Sepharose FastFlow (GE healthcare) according to manufacturer's protocol. After washing with Phosphate Buffered Saline (PBS) plus 0.1% Tween-20 (PBS-T) to remove non-specific proteins, bound antibodies were eluted from the antigen column with 0.2 M Glycine (pH 2.0). Eluted antibodies were dialyzed with PBS containing 10% Glycerol and concentrated by centrifugal concentrator to 1mg/ml. Affinity-purified antibody was stored at 4°C with preservatives and is referred to as APC-M2 pAb.

Results

Intermediate Filament protein lamin B1 co-precipitates with APC using the new APC-M2 pAb

As a multi-functional tumor suppressor protein, APC has been widely analyzed regarding its subcellular localization and its interaction with other proteins. Most commercial antibodies, which were raised against either the N- or C-terminus of APC, recognize proteins other than APC (22, 33). This cross reactivity makes it difficult to obtain reliable data using many molecular and cellular techniques to study APC. Thus, we affinity-purified rabbit sera raised against a central domain of APC (amino acid 959-1338, Figure 4.1), referred to hereafter as APC-M2 pAb. Most of the following studies utilized HCT116βw cells that express only the wild-type β-catenin allele (34), unlike the parental HCT116 cells that contain one wild-type and one mutant β-catenin allele. APC-M2 pAb recognizes full-length APC migrating as a single band of ~310 kDa from HCT116βw cells and truncated mutant APC at ~150kDa from SW480 cells (Figure 4.2A). No band was detected from HCA46 cells which encode a severely truncated APC (amino acid 1-213). Both full-length and truncated APC were efficiently precipitated using APC-M2 pAb (Figure 4.2B). A Well established binding partner of APC, β-catenin, was also co-precipitated with full-length APC from HCT116βw cell lysates (Figure 4.2B)

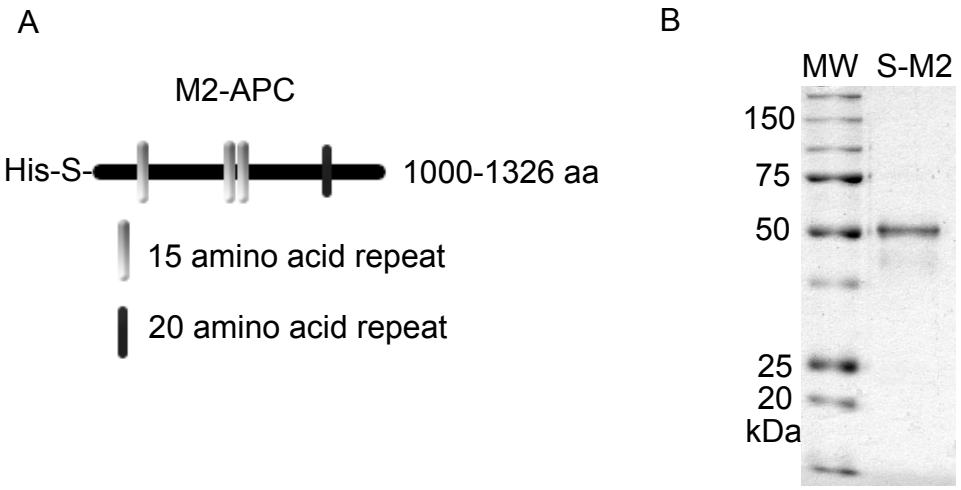

Figure 4.1
Generation of recombinant M2-APC immunogen. (A) Schematic diagram of the N-terminal His and S dual-tag fused APC fragment (amino acid 1000-1326) which contains the three 15 amino acid repeats and one 20 amino acid repeat. (B) Purified recombinant M2-APC fragment (~50kDa) used for immunization was resolved by SDS-PAGE and detected using colloidal blue.

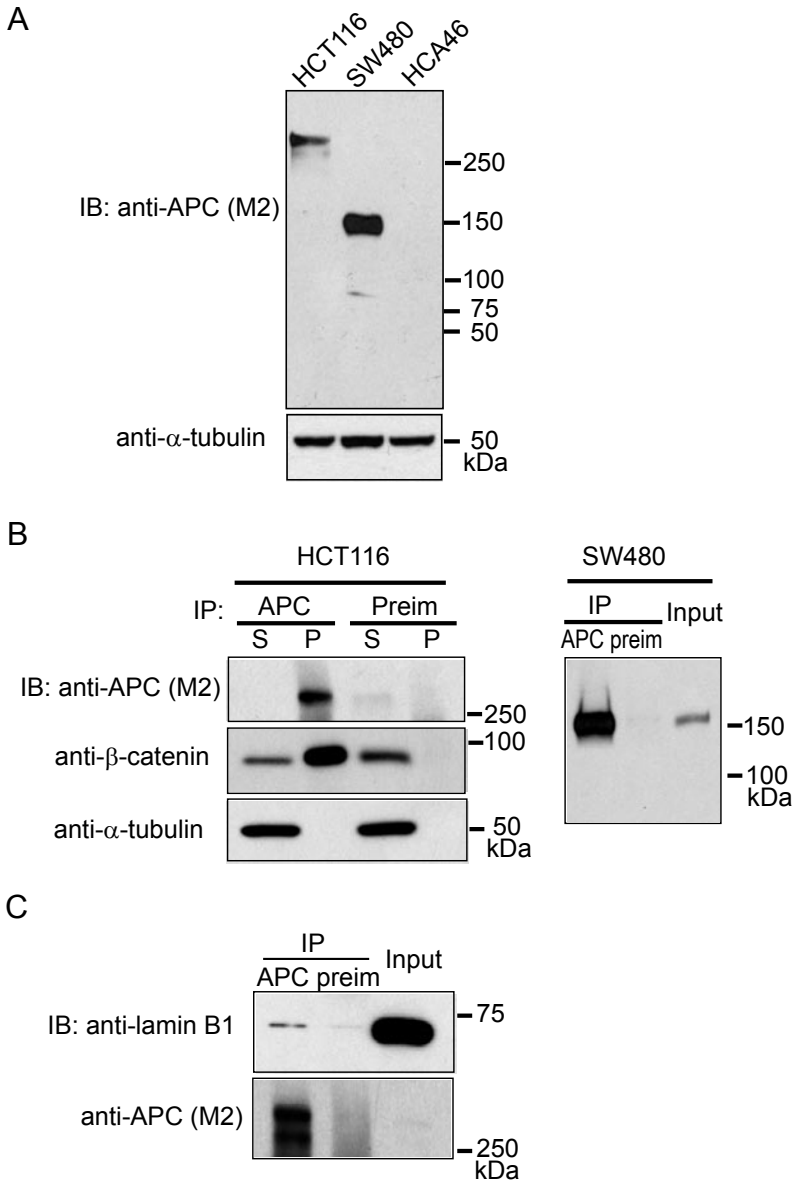

Figure 4.2
Lamin B1 co-precipitates with endogenous APC using APC-M2 pAb. (A) APC-M2 pAb specifically detects full-length (~310kDa) APC in HCT116βw cell lysates (lane 1) and truncated (~150kDa) APC in SW480 cell lysates (lane 2) by immunoblot. APC-M2 pAb does not detect the truncated APC (amino acid 1-213) in HCA46 cell lysates (lane 3). Equivalent total protein (30 μg) was resolved in each lane. Blot shows entire spectrum of proteins from > 300 kDa to ~ 15 kDa. α-tubulin is shown as a loading control. (B) APC-M2 pAb immunoprecipitates APC from HCT116βw (left panel) and SW480 (right panel) cell lysates. β-catenin co-precipitates with full-length APC (left panel, middle blot). Neither APC nor β-catenin co-precipitated with preimmunue sera (Preim). P, precipitated proteins; S, 10% of non-precipitated supernatant proteins (27μg). Input, 5% of total input proteins (25μg). (C) Endogenous lamin B1 was co-precipitated with APC using APC-M2 pAb, but not with preimmune sera. Input, 6% of total input proteins (37.5 μg). The lower band in the APC IP lane represents degradation products.

Thus, we confirmed the reliability of APC-M2 pAb for use in immunoblotting, and immunoprecipitation.

APC is a multi-functional protein involved in several different cellular pathways. Many of APC's functions, such as in cytoskeletal regulation, are only beginning to be elucidated. Identifying novel binding partners for APC is a step toward fully understanding APC's involvement in maintenance of cellular integrity. Therefore, APC-M2 pAb was used to co-immunoprecipitate APC and its associated proteins from HCT116βw cell lysates. Nine protein bands that were unique to the APC co-precipitation but not seen using preimmune sera were visualized by colloidal blue staining (Figure 4.3). These bands and corresponding regions from the control lane were separately isolated, pooled and analyzed by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) as described in Methods. Forty-three potential APC binding proteins, which have cross-correlation scores (x-corr) of $\geqq 2$ for 2+ ion and $\geqq 2.5$ for 3+ ion, were found following procedures described in Methods (Table 4.1). These 43 proteins could be grouped in eight broad categories, the largest two being cytoskeletal regulation and RNA processing/translation. From the most abundant potential APC-binding proteins found, we chose to examine the interaction of APC with intermediate filaments in more detail. IF proteins identified were lamin B1, lamin B2, keratin 81 and keratin 82 (Table 4.2). Initially, we focused on lamin B1, the most abundantly APC-associated

protein of the four. Interaction of lamin B1 with APC was verified by co-precipitation (Figure 4.2C).

Lamin B1 colocalization with APC in both cultured cells and human colonic tissue revealed using APC-M2 pAb

In order to validate the interaction of lamin B1 with APC using immunofluorescence, we first verified the utility of APC-M2 pAb for immunofluorescence microscopy. APC-M2 pAb revealed APC at cell-cell junctions, in the nucleus and in the cytoplasm of HCT116βw cells (Figure 4.4A). This pattern partially overlapped that of one of the most reliable commercially available APC antibodies, ab-7, which was raised against amino acid 1-266 (22). The most intense areas of staining within HCT116βw cells (Figure 4.4A) resembled localization at the centrosome previously reported by Olmeda and Henderson (35, 36). In SW480 cells in particular, APC-M2 pAb revealed more immuno-reactivity to APC in the nucleus compared to that of ab-7, the pattern of which was described previously (27). Importantly, APC-M2 pAb did not produce any signal in HCA46 cells which are predicted to express APC protein lacking the M2 region (Figure 4.4B). The slight fluorescent signal seen in HCA46 cells stained using APC-M2 pAb was similar to that of control cells stained with only secondary antibody (data not shown). Using APC-M2 pAb, we found partial overlap in the staining pattern of APC with IF proteins lamin B1 and keratin (Figure 4.4C).

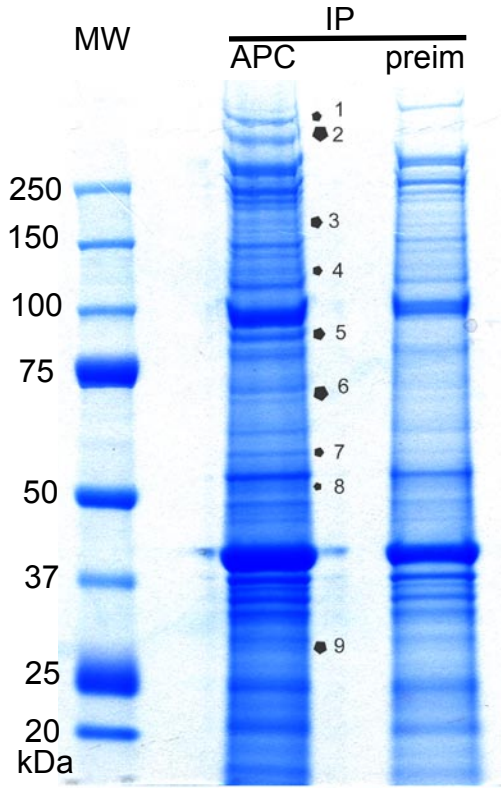

Figure 4.3
APC-M2 pAb co-precipitates APC binding proteins. Proteins co-precipitated from HCT116βw cell lysates using APC-M2 pAb were resolved on a 4-12% NUPAGE gel followed by colloidal blue staining. Stars mark the 9 protein bands that were precipitated using the APC-M2 pAb and not using preimmune sera.

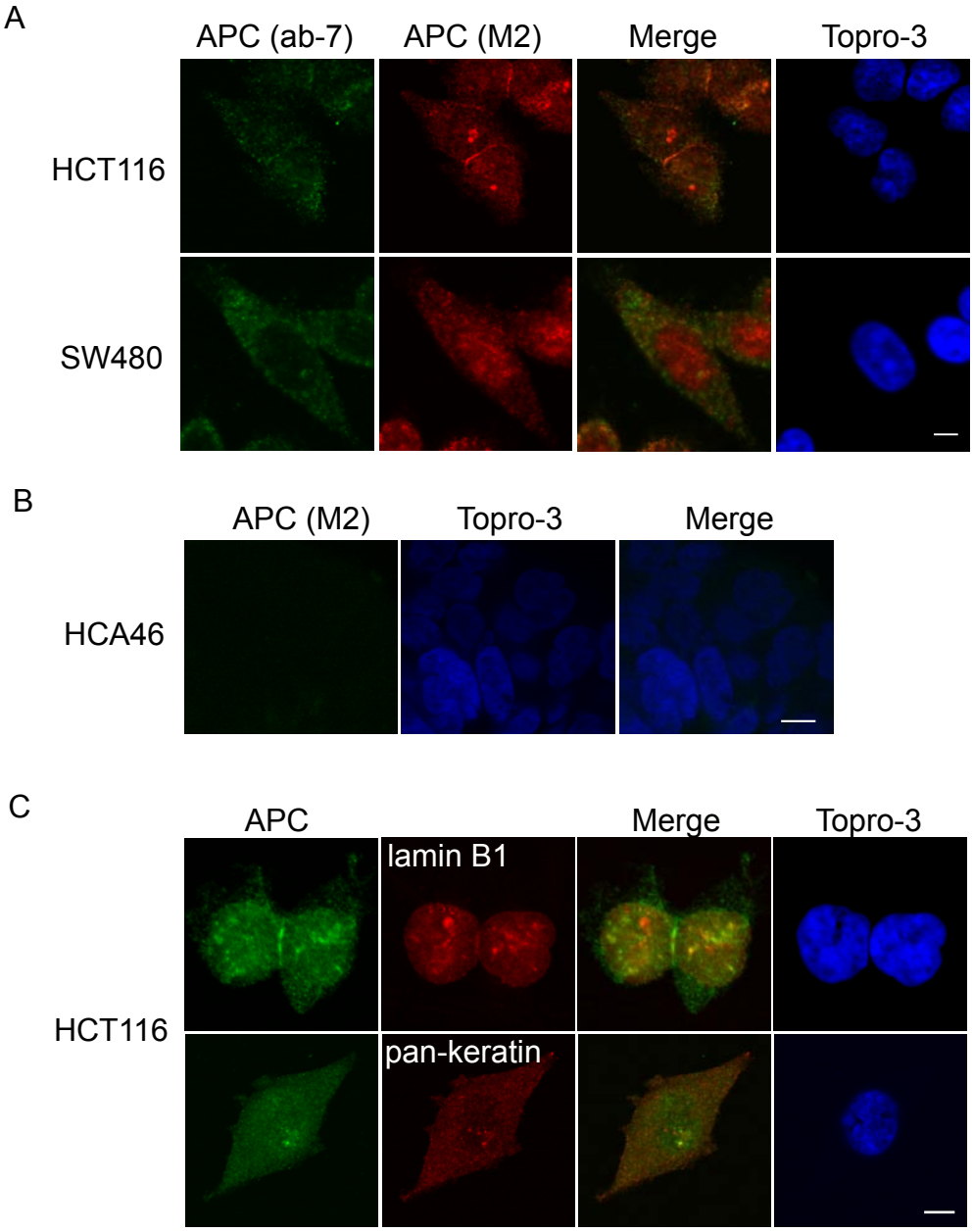

Figure 4.4
APC colocalizes with IF proteins lamin B1 and keratins in cells. (A) Confocal immuno-fluorescence microscopy of HCT116βw and SW480 cells double-labeled with APC-M2 pAb (red) and commercial anti-APC (ab-7) mAb (green). Scale bar, 5μm. (B) Confocal immunofluorescence microscopy using APC-M2 pAb to probe HCA46 cells expressing N-terminally truncated APC (amino acid 1-213) reveals no signal. Scale bar, 5μm. (C) Confocal immunofluorescence microscopy of double-labeled HCT116μw cells reveals that a subset of APC (green) protein colocalizes with lamin B1 (red) and keratins (red). Scale bar, 5μm.

Table 4.1 Full list of proteins associated with APC as identified with LC-MS/MS

Protein	Accession No.	Peptides found 1st / 2nd run	Functional category
Adenomatous polyposis coli	P25054	89/78	N/A
Lamin B1	Q6DC98	7/6	Intermediate filament
Lamin B2	Q03252	2/3	
Keratin 81	Q14533	5/5	
Keratin 82	Q9NSB4	2/2	
α-actinin-4	043707	4/3	Cytoskeletal regulation
WD repeat protein 1	075083	3/3	
Epiplakin	P58107	3/2	
Protein phosphatase 1 regulatory subunit 12A	014974	3/2	
Tubulin α-ubiquitous chain	P68363	3/2	
Tubulin β chain	P07437	3/2	
Tropomyosin-3	P06753	2/2	
Neurabin-2	Q96SB3	2/2	
α-actinin-1	P12814	2/1	
β-catenin	P35222	70/67	Transcription
RuvB-like 2	Q9Y230	7/8	
RuvB-like 1	Q9Y265	6/6	
RNA-binding protein 14	Q96PK6	6/7	
Ret finger protein isoform β variant	Q59EC6	4/2	
Heterogeneous nuclear ribonucleoprotein K	P61978	3/5	
Pescadillo homolog 1	000541	2/2	
Protein TFG	Q92734	8/6	Chromosome rearrangement
Ladinin 1	000515	8/8	Extracellular matrix
40S ribosomal protein S4, Y isoform 1	P22090	8/5	RNA processing & translation
ATP-dependent RNA helicase DHX15	043143	7/9	
Splicing factor arginine/serine-rich 1	Q07955	6/4	
Heterogeneous nuclear ribonucleoprotein M	P52272	4/3	
ATP-dependent RNA helicase DDX3X	000571	4/2	
Nucleolar RNA helicase 2	Q9NR30	3/2	

(Table 4.1 continued)

Splicing factor 3b, subunit 3	Q6NTI8	2/3	
Heterogeneous nuclear ribonucleoprotein Q	O60506	2/2	
Nuclear fragile X mental retardation-interacting protein 2	Q7Z417	3/3	
Transducin beta-like 3 variant	Q59GD6	2/2	
Heat shock 70 kDa protein 2	P54652	7/6	Chaperones
Heat shock 70kDa protein 5	P11021	4/2	
Liprin-β1	Q86W92	3/5	Tumorigenesis
LRCH3 precursor	Q96II8	5/4	Others
Transketolase	P29401	3/3	

43 proteins; each peptide of which passed Xcorr cutoffs of 2 [+2 ion], 2.5 [+3 ion]; for all proteins at least 2 peptides found each duplicate run

Table 4.2 Intermediate filament proteins associated with APC as identified with LC-MS/MS

Protein	Accession No	Peptides found 1^{st} / 2^{nd} run
Lamin B1	Q6DC98	7/6
Lamin B2	Q03252	2/3
Keratin 81	Q14533	5/5
Keratin 82	Q9NSB4	2/2

at least 2 peptides found in each duplicate run; all peptides passed Xcorr cutoffs of 2 for +2 ion and 2.5 for +3 ion

In normal human colonic tissue, both APC and lamin B1 exhibit increasing expression from the bottom of crypts toward the luminal surface (Figure 4.5A-a). A higher magnification of the confocal image reveals perinuclear colocalization of APC and lamin B1 throughout the crypt (Figure 4.5A-b). Although APC staining in stromal cells was faint compared to in the epithelia, there appears to be some colocalization of APC with lamin B1 in the stromal cell nuclei located between the crypts. As a positive control, we co-stained normal human colonic tissue for β-catenin and APC (Figure 4.5B). APC-M2 pAb revealed APC in both nuclei and nucleoli, with a staining pattern partially overlapping that of β-catenin as previously described (13, 37).

APC association with intermediate filament proteins is not dependent on actin or tubulin

IFs interact with both actin-containing microfilaments and microtubules [see review (38)]. Because APC also associates with actin and microtubules, it is possible that the co-immunoprecipitation of IF proteins with APC was due to precipitation of a large complex of cytoskeletal components. To determine whether the APC/IF interaction was dependent on actin-containing microfilaments and microtubules, we performed a traditional solublization and extraction of both tubulin- and actin-containing microfilament proteins from cells, leaving behind predominantly insoluble IFs and desmosomes (30). After sequential extraction, the cytoskeletal proteins tubulin and actin were efficiently removed from cells as determined by immunofluorescence microscopy

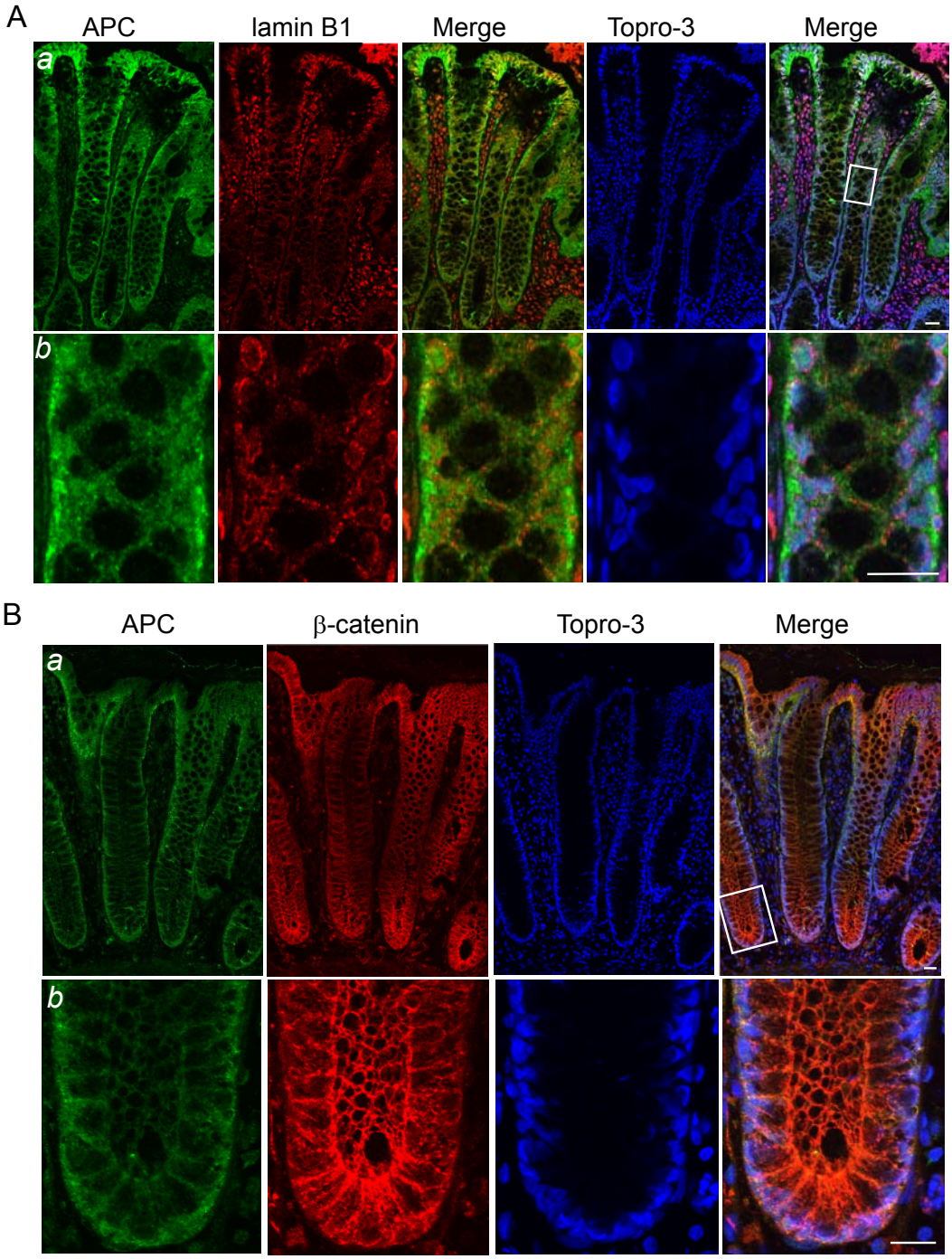

Figure 4.5
APC colocalizes with lamin B1 in human colonic tissue. Confocal immunofluorescence microscopy of cryosections from normal human colon tissue triple-labeled with APC-M2 pAb (green), DNA dye topro-3 (blue)and lamin B1 (red) (A) or β-catenin mAb (red) (B). Scale bars, 20 μm. Tissues are oriented so that luminal surfaces are at the top of the images. (b) Enlarged image of the region indicated in (a). (A) APC colocalizes with lamin B1 (red) around nuclei. (B) APC colocalizes with β-catenin in the cytoplasm and cell junctions of human colonic crypts.

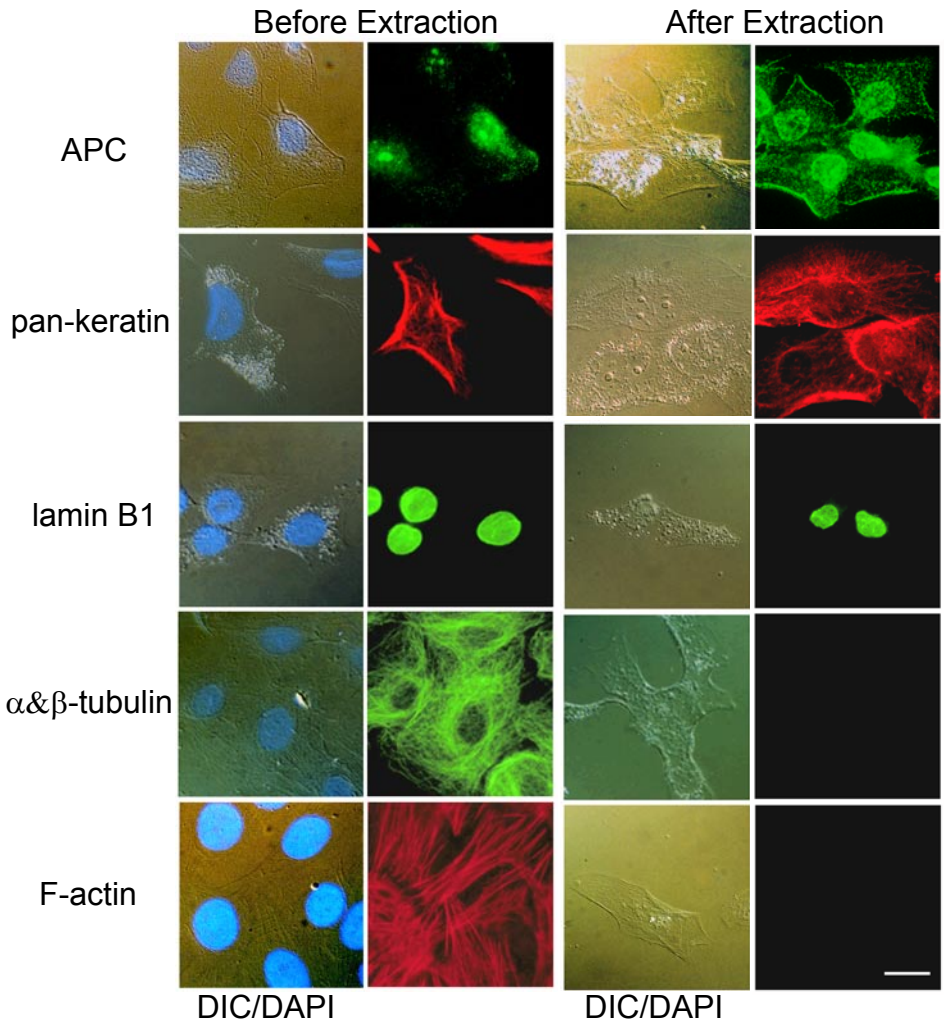

Figure 4.6
APC remains associated with IF proteins following extraction of actin and tubulin. Left two panels, 184A1 epithelial cells analyzed by immunofluorescence microscopy reveals normal distribution of APC, lamin B1, keratin, tubulin, actin (detected with phalloidin) and DNA (blue in DIC overlay). Right two panels, following sequential extraction, APC remained cell associated, along with IF proteins lamin B1 and keratin. Note that actin, tubulin, and DNA were successfully extracted from these cells. Nuclei are indicated by DAPI staining. Scale bar, 10μm.

(Figure 4.6). APC, however, remained associated with IFs as indicated by lamin B1 and pan-keratin staining. Finding APC still associated with cells following this extraction procedure suggests a stable interaction between APC and IFs, independent of actin-containing microfilaments and microtubules.

APC-M2 pAb is a versatile antibody, specific for APC

We have demonstrated that APC-M2 pAb specifically recognizes APC protein in cells by immunoblot (Figure 4.2A) and immunoprecipitation (Figure 4.2B-C and Table 4.1) and in both cells and human tissue by immunofluorescence confocal microscopy (Figure 4.4 and 4.5). Many mouse models have been established to analyze APC functions in the context of a whole mammalian organism. To validate the use of APC-M2 pAb in animal studies, frozen sections of mouse intestinal tissue were probed with the APC-M2-pAb and then APC was visualized using confocal microscopy. In the small intestine, APC appeared at cell-cell junctions and in the cytoplasm (Figure 4.7A and B). In enterocytes of the villus, cytoplasmic puncta were also visible, particularly near the basal surface (Figures 4.7B). APC staining was most intense in cells at the top of villi, with less APC in cells near the crypt base (Figure 4.7A). In the colon, APC-M2 pAb revealed APC in both nuclei and nucleoli, with a staining pattern partially overlapping that of β-catenin as previously described (Figure 4.7C and D) (13, 37). In some cells at

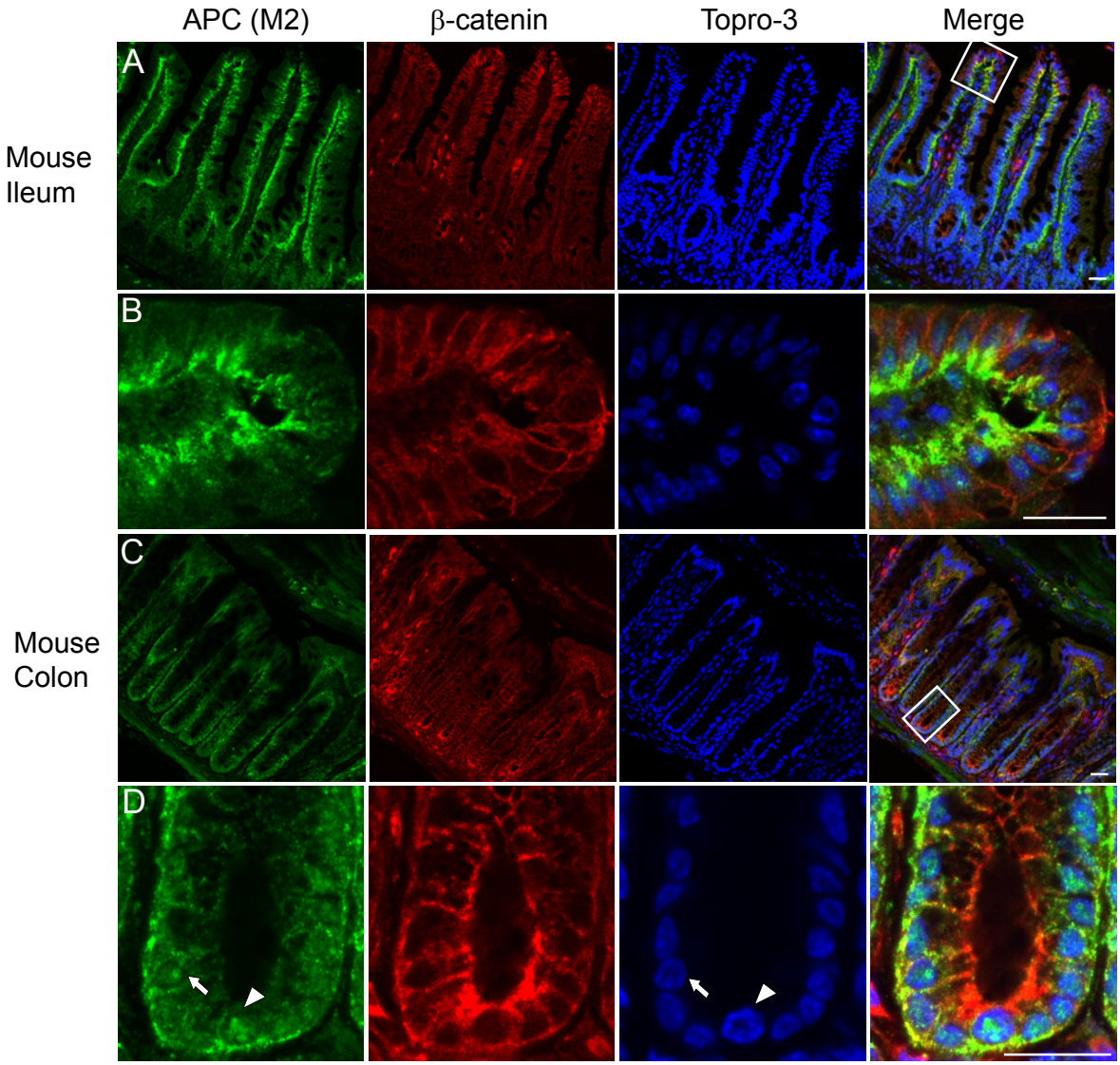

Figure 4.7
APC-M2 pAb recognizes endogenous APC protein in mouse intestinal tissue by immunostaining. Confocal immunofluorescence microscopy of cryosections from wild-type mouse ileum (A, B) and colon (C, D) tissues triple-labeled with APC-M2 pAb (green), β-catenin mAb (red) and DNA dye topro-3 (blue). Scale bars, 20 μm. Tissues are oriented so that luminal surfaces are at the top of the images. (A) There is an increasing gradient of APC staining from crypts to villi. (B) Enlarged image of a region at the villus tip shown in (A). APC staining is concentrated in basolateral regions of villus enterocytes. (D) Enlarged image of a region at the base a colonic crypt shown in (C). APC locates to cell junctions, cytoplasm, nucleus, and nucleoli (arrow). Arrowhead indicates spindle-like staining pattern.

the base of the crypts, the spindle-like distribution of APC suggested association with centrosomes and microtubule organization centers (Figure 4.7D). Of note, APC-M2 pAb did not reveal the apical APC distribution in intestinal tissues that has previously been attributed to cross reactivity with a non-APC protein (39).

Discussion

In the current study, we have generated an APC-M2 pAb that is specific for APC. It is versatile and can be used to detect APC in immunostaining, immunoblotting and immunoprecipitation. Using this antibody, we identified a novel interaction between APC and IFs. The APC and IF protein interaction was confirmed by co-precipitation and colocalization in cultured epithelial cells and human intestinal tissue. Association of APC with IFs is not dependent on actin-containing microfilaments and microtubules.

APC mutations are found in over 80% of colorectal cancers and have been assessed for screening and prognosis purposes in clinical settings. For such research, access to an APC antibody that accurately depicts levels and subcellular localizations of APC in cells and especially in tissues is extremely important. According to Wakeman et.al (22), many commercially available antibodies also recognize an unidentified protein that localizes to the apical region of polarized epithelial cells. Most currently available commercial APC antibodies were raised against several hundred amino acids at either the

N- or C-terminus of the APC protein. However, most of these antibodies have not been proven to be specific for APC (22). Furthermore, most of these antibodies are only useful for a single application. For example, anti-APC Ab-7 is only effective when used for immunostaining. Therefore, we generated a new antibody against the 15 amino acid repeat region in the middle of APC (Figure 4.1). This affinity-purified APC-M2 pAb recognizes junctional, cytoplasmic and nuclear APC by immunostaining (Figure 4.4A) without producing the non-specific apical staining pattern previously seen (22).

One of the most clinically relevant applications for APC antibodies is for immunohistochemical analysis of tissues. We have validated the use of APC-M2 pAb in the immunostaining of sections of frozen human colonic tissue and mouse intestinal tissue. Previously, APC has been detected in both the nucleus and cytoplasm of human colonic tissue, with more prevalent cytoplasmic staining in colonic tumors compared to normal tissue (37). In our study of human colon tissue, APC-M2 pAb revealed APC in both nuclei and at cell-cell junctions, partially colocalized with β-catenin, similar to previous reports (13, 37) (Figure 4.5A). APC protein also appeared concentrated near the basal surface of epithelial cells (Figure 4.5A-a and B-a) as previously reported (40). In the mouse colonic tissue, the spindle-like distribution of APC we observed in some cells near the bottom of a crypt (Figure 4.7D) has been previously reported only in cultured cancer cell lines (35, 36). APC staining became most intense in cells at the top of villi in

the mouse small intestine (Figure 4.7A) and in cells at the luminal surface of the human colon (Figure 4.5), consistent with a role for APC in differentiated cells. Most importantly, APC-M2 pAb did not recognize any protein at the apical surface of intestinal tissues. Thus, APC-M2 pAb does not appear to exhibit the cross reactivity previously reported for some commercially available APC antibodies (39). Taken together, the APC-M2 pAb is a useful antibody for future examination of APC in mouse and human tissue sections. Future analysis of formalin-fixed paraffin-embedded tissues may uncover more clinical uses for this antibody.

Intermediate filaments are essential components of cytoskeletal structure. Recent reports have implicated APC in the regulation of cellular structural integrity through affects on microtubule growth directionality (41) and polarity (42). APC also associates with microtubule-interacting protein EB1 (17), and actin-associated protein ASEF (11). Our own previous screen for APC-interacting proteins identified keratin 18 (Stecklein and Neufeld, personal communication). Although classified as a hair cuticle-specific keratin, keratin 81 is expressed in a wide variety of tissues including mammary gland and lung (43-45) and in human embryonic kidney 293T cells (46). Keratin 82 is also thought to be expressed predominantly in hair and nail. However, when Flag tag specific antibodies were used to immunoprecipitate flag-tagged human homolog 3 of Drosophila Disk-large (hDlg) from human embryonic kidney 293 cells, keratin 82 was identified as a

protein partner (47). This observation is significant because it indicates that keratin 82 has a broader expression pattern than initially suggested and because hDlg protein itself is a binding partner of APC (48).

As a vital part of the cytoskeleton, intermediate filaments are dynamic and can stabilize cellular organelles such as the nucleus (49). Mutations in genes encoding IF proteins are associated with a number of diseases such as Monilethrix (K81) (50), Autosomal dominant leukodystrophy (LMNB1) (51), acquired partial lipodystrophy (LMNB2) (52), and various other lipodystrophies and cardiomyopathies (19). APC potentially cross-links IFs to improve their stability or binds IFs to other structures such as actin filaments or microtubules. Therefore, truncated APC found in the majority of colorectal cancers and many other cancers, might have compromised ability to link APC binding partners with different IF proteins. Further study of this novel APC/IF interaction might illuminate the underlying mechanisms of intermediate filament protein-associated diseases.

Conclusions

In summary, we identified an interaction between APC and intermediate filament proteins lamin B1 and keratin using a new antibody, APC-M2 pAb. The APC-M2 pAb antibody is specific for APC, versatile, and reliable, with potential value in the clinical

setting and in translational research. By sequential extraction of cytoskeletal components, we have shown that the association of APC with IF proteins is not dependent on actin and tubulin. Finding APC associated with IF proteins confirms and expands upon the previous notion that APC is in close association with the cytoskeleton, raising the possibility that APC functions in the maintenance of cytoskeletal structure and integrity.

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CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

As the key regulator to suppress tumorigenesis in colorectal cancers, APC has been extensively studied since its discovery in the early 1990s (1, 2). In addition to its traditional role as a negative regulator of Wnt signaling, other functions of APC have been increasingly emphasized. Despite the large body of work identifying these non-traditional roles for APC, most of these functions are only beginning to be understood. Therefore, identification of APC binding partners is a powerful approach to obtain functional information about APC in multiple pathways. In this work, I identified two novel binding partners of APC: topo IIα and lamin B1. Topo IIα mediates APC's function in the regulation of G2-M cell cycle transition; whereas lamin B1 might be regulated by APC, thus APC being involved cytoskeletal organization. In chapter 2 & 3, I described two regions in APC that specifically bind topo IIα but not topo IIβ. Expression of either domain dramatically affects the activity of topo IIα *in vitro* and *in vivo* and delays cell cycle progression in G2, but only in cells with normal level of topo IIα. For the first time, we report a β-catenin independent role for APC in the regulation of G2-M transition.

I propose that the G2 cell cycle arrest results from inhibition of topo IIα leading to mis-regulation of DNA untangling. This inhibition of topo IIα activity seems to occur with either M2- of M3-APC, amino acid 959-1338 or 1211-2073, respectively. Most APC mutations found associated with colorectal cancers result in truncation of APC protein between amino acid 1250 and 1450 (3). Thus, most of M3- and even part of M2-APC are lost in colorectal cancers. It will be interesting to determine if truncated APC also inhibits topo IIα activity similar to M2- and M3-APC. One approach to analyze this potential is to generate a GFP-fused APC_{1417} (amino acid 1-1417), a region commonly used in mammalian systems to mimic truncated APC in human cancers. The topo IIα decatenation activity could then be evaluated in nuclear extracts from GFP-APC_{1417} expressing cells. Along the same line, it will also be informative to analyze the effect of expression of M2- and M3-APC in colonic cancer cell lines with truncated APC, such as SW480 and Caco2 cells. Will introduction of M2- or M3-APC also cause G2 cell cycle arrest and inhibition of topo IIα activity in nuclear extracts from those cell lines?

M2- and M3-APC enhanced topo IIα decatenation activity in an *in vitro* system using purified recombinant proteins, but inhibited topo IIα activity in nuclear lysates from transfected cells. The different responses potentially stem from lack of post-translational modifications in the *in vitro* system. Phosphorylation of APC has been implicated in β-catenin down-regulation, APC nuclear transport and other cellular

functions (4-8). Recombinant M2- and M3-APC proteins produced in and purified from bacteria are not phosphorylated. This lack of modification might alter the ability of M2- and M3-APC to interact with topo IIα and thus their ability to modify topo IIα activity. On the other hand, topo IIα has been reported to be phosphorylated, ubiquitinated and even SUMOylated in cells (9-12). However, the exact consequence of these modifications is not clear. It is possible that APC is involved in some of these topo IIα modifications. Therefore, M2- or M3-APC expressed in cells would efficiently mediate post-translational modifications of topo IIα, while purified M2- or M3-APC would not. In this scenario, regents to identify specifically modified topo IIα or APC will need to be developed to facilitate testing of this hypothesis. Alternatively, identifying the effect of endogenous full-length APC on topo IIα activity will also be illuminating. Inefficient transfection of mammalian cells with expression constructs for full-length APC has been an obstacle to exploring this further. Therefore, developing a stable cell line with inducible expression of full-length APC will be beneficial as well.

Inhibiting topo IIα initiates the G2 decatenation checkpoint through ATR kinase and cyclin B1 (Figure 1.7) (13). Based on the inhibitory effect of M2- and M3-APC, I hypothesize that full-length APC has an inhibitory effect on topo IIα as well (Figure 5.1). I predict that full-length APC either directly binds to topo IIα to suppress its activity or recruits other factors that restrain its activity. These inhibitory signals could be proteins

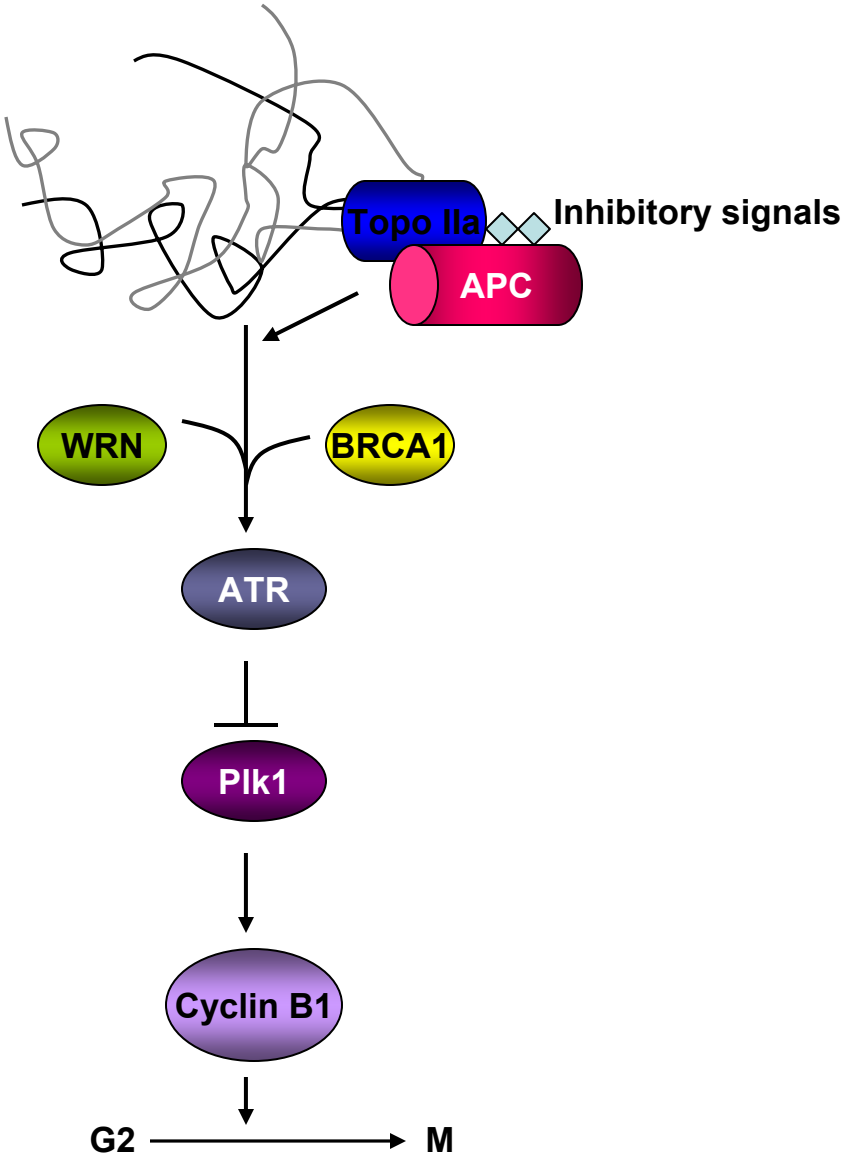

Figure 5.1

Working model of APC's role in the regulation of the G2 decatenation checkpoint. If the chromosomes are not properly detangled before entering M phase, ATR kinase can be activated to initiate the decatenation checkpoint. In this process, APC binds to topo IIα, recruits proteins that directly inhibit topo IIα, or brings in molecules that modify topo IIα, thus inhibiting topo IIα activity. This inhibition activates the G2 decatenation checkpoint, giving the cell time for DNA detangling or further cell fate determination. (Modified from Damelin and Bestor, 2007)

that directly suppress topo IIα activity, or intermediate molecules, such as ubiquitin ligase, that execute post-translational modifications of topo IIα. Future efforts to characterize the components of the APC-topo IIα complex will test this model.

As the key tumor suppressor in the pathogenesis of colorectal cancer, APC has become one of the most intensively studied proteins in both basic and clinical research (14-16). Lack of reliable regents to analyze APC expression in both cell and tissue studies is a rate limiting step, which holds back translational research. Although progress has been made with antibody-based investigations, many commercial available APC antibodies non-specifically recognize proteins other than APC (17, 18). This questionable antibody specificity adds confusion to the APC research field making interpretation of a number of publications regarding APC difficult. Therefore, I generated a specific and reliable antiserum, APC-M2 pAb, which will serve as a valuable reagent for future immuno-based studies on APC and APC-related colorectal cancers.

This new APC-M2 pAb is also an efficient tool to identify APC binding proteins. By co-immunoprecipitation, I discovered novel interactions between APC and intermediate filament proteins. Chapter 4 described characterization of this new APC-M2 pAb antibody, as well as identification of the novel APC/intermediate filaments protein interaction using APC-M2 pAb for immunoprecipitation. Actin filaments, microtubules meshwork and intermediate filaments (IFs) are the three types of cytoskeletal components

that collaborate to regulate epithelial integrity, polarity and migration. As a scaffold to maintain cell and tissue integrity, defects in IFs impact a number of diseases [see review (19)]. Therefore, identification of an interaction between APC with IF proteins might help us understand the underlying mechanisms of IF-associated diseases. I speculate that APC serves as a bridge to connect actin filaments, microtubules and intermediate filaments thus stabilizing epithelial organization. Inactivation of the APC by truncated mutation or hypomethylation de-regulates the cytoskeleton by over or under cross-linking filaments. Hence, it will be interesting to determine if APC still interacts with IF proteins in colonic cancer cells possessing truncated APC. Further functional analysis is required to illuminate the exact physiological contribution of APC in cytoskeleton integrity.

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APPENDIX CHAPTER

CONDITIONS FOR EXTRACTION AND IMMUNOSTAINING OF MOUSE INTESTINAL TISSUES

Introduction

In the investigation of colorectal cancers, many mouse models have been established and widely studied. Immunohistochemistry (IHC) and immunochemistry (IC) of mouse intestinal tissues have been intensively used in pathologic studies. Paraffin embedded sections are the best preservation method. However, the antigen retrieval step does not always recover the antigen epitopes that are quenched during the complicated embedding procedure. Cryo-sections, although better preserving antigen epitopes because of less harsh experimental conditions, still have limitations in revealing some antigens. Therefore many large antigens that are physiologically unstable, such as APC, can not survive the complicated procedures of IHC and IC on paraffin- or cryo-tissues. Further, sectioning of tissue, such as colonic tissue, sometimes does not fully retain tissue structure, thus cannot give three-dimensional structures. This shortcoming prevents us from obtaining structure-related protein distribution in tissue. Thus, we developed an immunofluorescence-based method to stain paraformaldehyde (PFA) fixed manually micro-dissected intestinal tissues. In the following text, we record the conditions of

micro-dissection of mouse intestinal villus and crypts, followed by immunofluorescence staining using a few specific commercial antibodies including anti-APC.

Materials and Methods

Isolation of mouse intestinal tissues

Mice are euthanized by CO_2 asphyxiation. Colon and small-intestine tissues removed carefully and washed thoroughly with ice-cold PBS. Tissues were fixed with 4% PFA on ice for 30min on a piece of Parafilm. Fixed tissue should be stored in PBS at 4° C for less than a week to achieve best results.

Micro-dissection was performed using a regular dissection microscope. A piece of tissue comprised of 10-30 crypts/villi was peeled from the colon/small-intestine tissue using the finest micro-dissection quality tweezers. This tiny piece was placed in PBS on a glass slide and viewed with the dissection microscope. Single crypt/villus structures were isolated carefully using a fine needle made from super fine wires. Isolated crypt/villus were then transferred carefully to a 0.5ml eppendorf tube containing 300µl of PBS. Tissues were kept on ice during the dissection procedure. Each sample for immunofluorescence staining should contain at least 50 crypts/villi. Isolated crypts/villi should be kept on ice for 48hrs or less before staining to achieve best results.

Immunofluorescence

Micro-dissected villi or crypts in 300µl of PBS were first permeabilized by 15µl of 10% Triton X-100/PBS (final concentration of 0.5%) for 1hr at room temperature (RT) with gentle agitation. Crypts/villi were collected by spinning at 150Xg for 5min. Then crypts/villi were blocked with 200µl MOM Concentrate (VECTOR LABORATORIES) overnight at 4° C with gentle agitation, if primary antibodies were made in mouse or rat. Otherwise, crypts/villi were blocked in 200 µl of 5% normal goat serum (NGS) (Sigma), 1% bovine serum albumin (BSA) (Sigma) and 0.1% Triton X-100 in PBS overnight at 4 ° C with gentle agitation. Crypts/ villi were then collected by spinning at 150Xg for 5min. Crypts/villi were then incubated with primary antibodies diluted in 200µl of MOM Diluent (VECTOR), if using MOM blocking procedure, or in 200µl of blocking buffer, for 24 hrs at 4° C with gentle agitation. Crypts/ villi were then collected by spinning at 150Xg for 5min. Then, 300-400 µl of TBS-T was added to the tube. All contents in the tube were then poured into a 15ml conical tube. Repeat this step until all crypts/villi are transferred into the conical tube. The final volume of TBS-T was then added to 10ml. This washing step should be carried out at 4° C with gentle agitation for 24 hrs. Crypts/ villi were then collected by multiple spinning (3-4) and then transferred to a new 0.5ml eppendorf tube. Crypts/villi were incubated with Alexa-fluorophore conjugated-secondary antibodies overnight at 4° C with gentle agitation in dark. If using

primary antibody made in mouse or rat, the secondary antibodies should be pre-absorbed with whole-mount intestinal tissue before applying to samples. The washing step and spinning steps are essentially the same as that after primary antibody staining. Topro-3 (1:500, Invitrogen) is used to co-stain crypts/villi for 5min RT. At last, mount crypt/villus with Prolong (Invitrogen) on a waterproof pap-pen- (Invitrogen) marked glass slide. Confocal Microscopy should be performed within a week for best signals.

Antibodies

The following antibodies are used for immunostaining: anti-APC (ab-1, 1:50, Oncogene), anti-APC (ab-7, 1:50, Oncogene), anti-APC (ali12-28, 1:2000, Chemicon), anti-APC (F3, Oncogene, 1:200), anti-β-catenin (1:2000, Sigma), anti-Musashi-1 (1:200 R&D), anti-Ki67 (1:200, DAKO), anti-BrdU (1:50, BD Bioscience), Alexa488-conjugated Wheat germ agglutinin (1:2000, Invitrogen), anti-Chromogranin A (1:100, Abcam), Alexa568-conjugated Phalloidin (1:500, Invitrogen) and anti-Lysozyme (1:100, Sigma).

Results and Discussions

Colocalization of APC with β-catenin in mouse colonic tissues

β-catenin is one of the most important and the most extensive studied binding partners of APC. We examine the colocalization of APC with β-catenin in the mouse colonic crypt isolated by manual micro-dissection. APC specific antibody ab-7 is used, because it is considered the most reliable commercial available APC antibody for immunostaining (1). APC predominately colocalizes with β-catenin at cell-cell junction and cytoplasm (Figure app.1.1).

Immunofluorescence of micro-dissected intestinal tissues has been one of most reliable approach to assess APC protein expression by commercial APC antibodies in our experience. PFA requires critical conditions to achieve its optimal effect and can be reversed if stored at 4C for a long time. However, this is the best method so far working for APC–related immunostaining. Since the procedure lasts less than a week, the antigens are usually well preserved thus ensuring the antibody efficiently recognizes the antigen. Also, the manual micro-dissection also guarantees the integrity of the full structure of intestinal tissues, such as a small intestine villus or a colon crypt.

Molecular markers for cell proliferation and differentiation in mouse intestine

Loss of APC has been shown to perturb intestinal enterocytes proliferation and differentiation (2). APC has also been implicated in stem cell proliferation and differentiation (3). In mouse model studies, using cell type specific markers to evaluate cell proliferation and differentiation has been widely accepted. Here I describe molecular markers that specifically detect intestinal stem cells (using anti-Musashi-1) (Figure app.1.2), proliferative cells (anti-Ki67) (Figure app.1.3), and the three major cell types in the intestine: goblet cell (Wheat germ agglutinin-WGA) (Figure app.1.4), paneth cell (anti-Lysozyme) (Figure app.1.5) and enteroendocrine cell (anti-Chromogranin A) (Figure app.1.6). Although Musashi-1 has been proposed to be one of the intestinal stem cell markers (4), its specificity has been controversial and needs further validation.

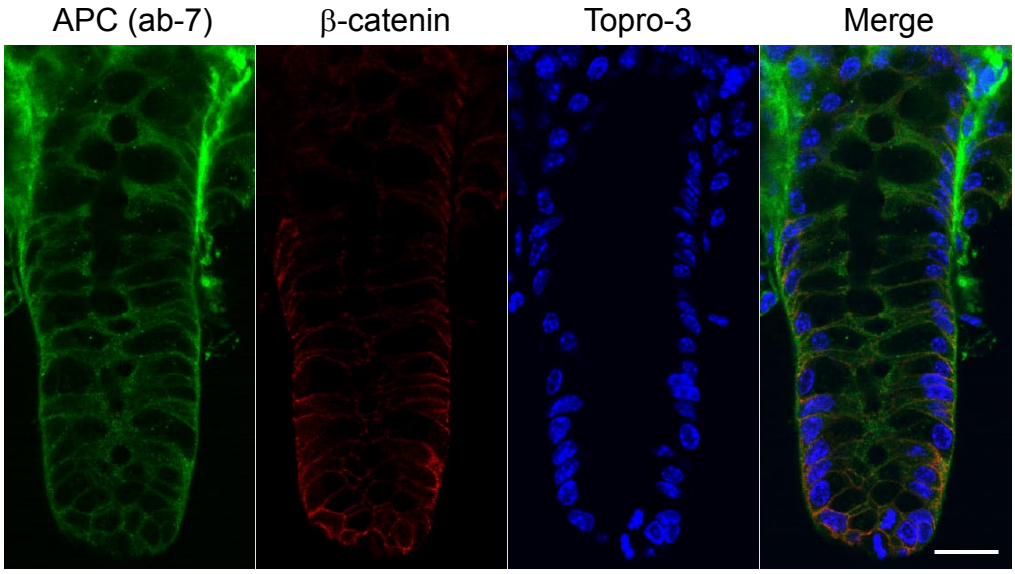

Figure app.1.1
APC (shown in green) and β-catenin (shown in red) specific antibodies recoganize endogenous proteins in a mouse colonic crypt. APC and β-catenin colocalizes predominantly at cell adhesion junctions. Scale bar, 20μm.

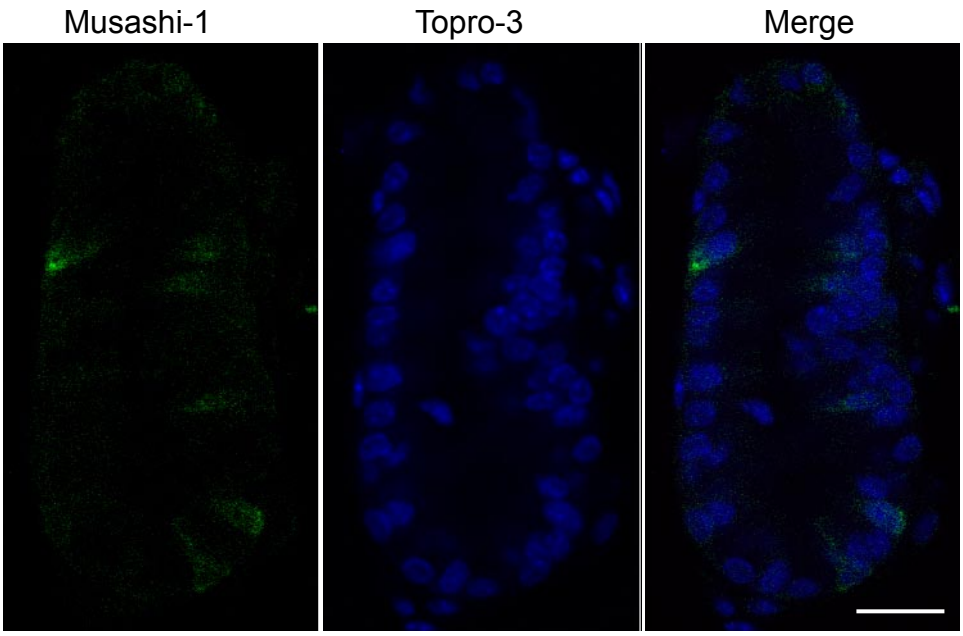

Figure app.1.2
Mushashi-1 specific antibody (shown in green) indicates colonic stem cell/TA cells in a mouse colonic crypt. Mushashi-1 predominantly localizes in the cytoplasm. Scale bar, 20μm.

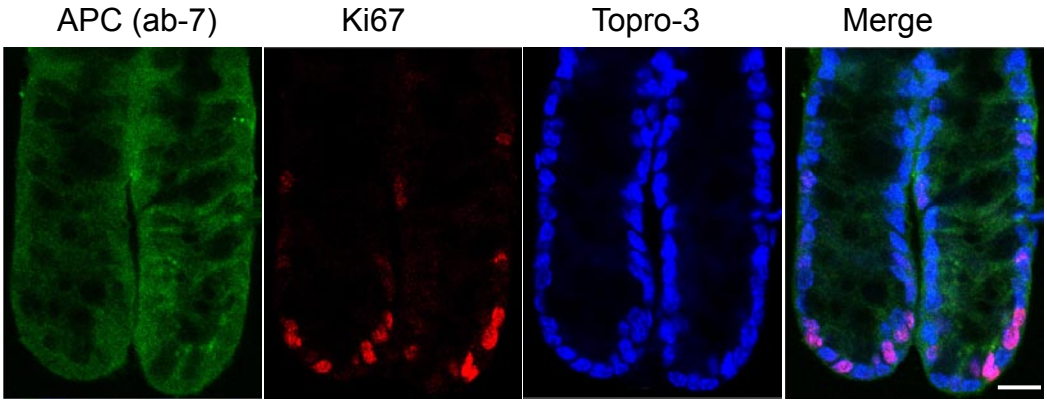

Figure app.1.3
Ki67 specific antibody (shown in red) specifically marks colonic cells that are proliferative in a mouse colon crypt. Ki67 predominantly localizes in the nucleus. Scale bar, 20μm.

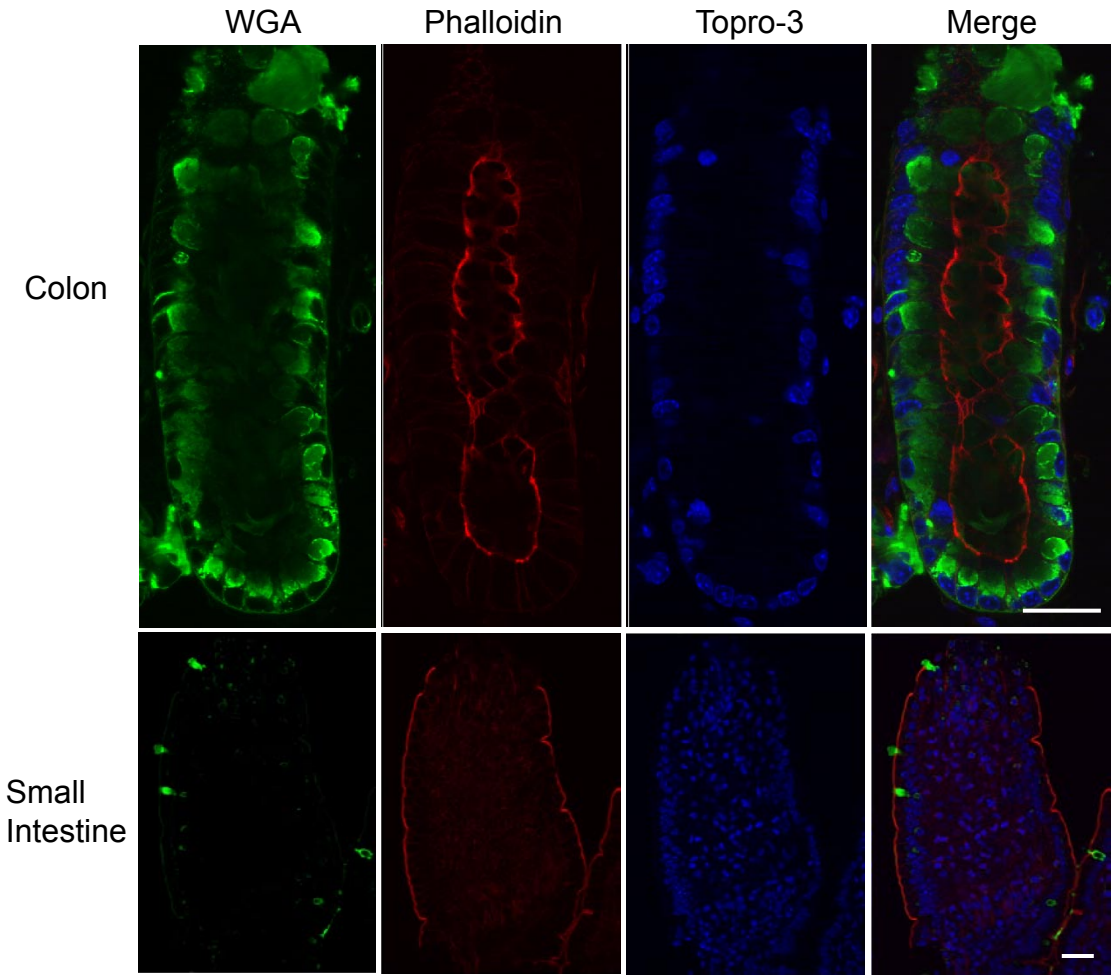

Figure app.1.4
WGA (shown in green) specifically marks goblet cells in a mouse colon crypt by staining mucin secreated by these goblet cells. Phalloidin outlines F-actin. Scale bar, 20μm.

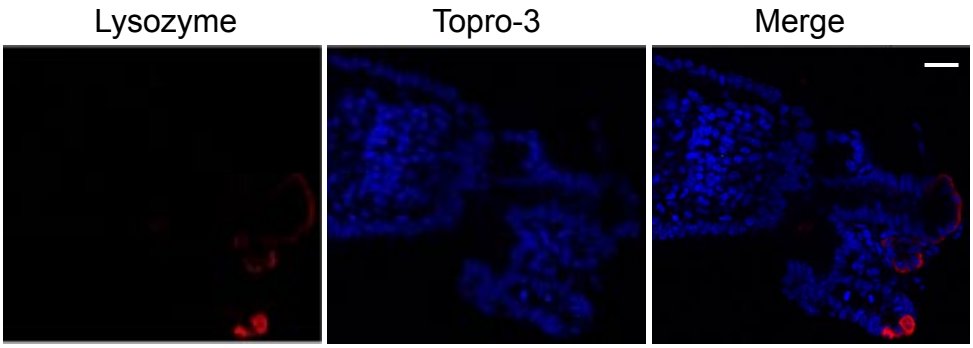

Figure app.1.5
Lysozyme specific antibody (shown in red) marks paneth cells in the crypt region of the small intestine. Note that paneth cells do not exist in colon. Scale bar, 20μm.

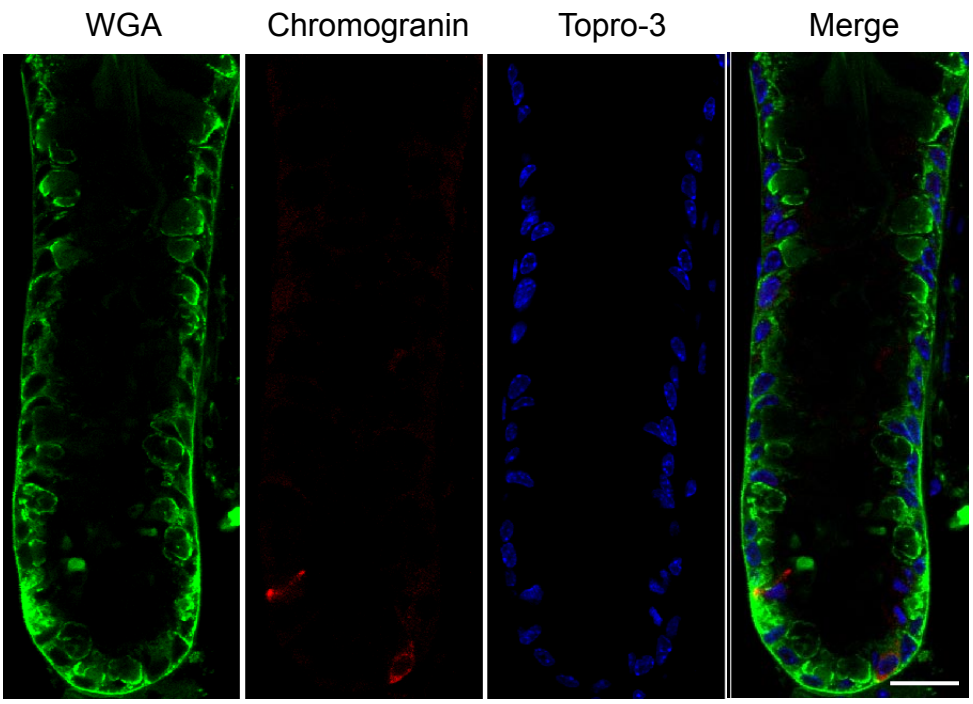

Figure app.1.6
WGA (shown in green) and chromogranin specific antibody (shown in red) mark goblet cells and enteroendocrine cells, repectively in a mouse colon crypt. Scale bar, 20μm.

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